

MOLECULAR CHARACTERIZATION OF
CONFORMATIONAL INTERMEDIATES IN THE UNFOLDING AND
DISSOCIATION OF BOVINE PLASMA AMINE OXIDASE

by

CALEEN B. RAMSOOK

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of
the requirements for the degree of Doctor of Philosophy,
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This manuscript has been read and accepted by the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the Degree of Doctor of Philosophy.

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Abstract

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By

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Advisor: Professor Lesley Davenport

Improperly folded proteins have been implicated in several diseases and an understanding of the pathways that lead to the formation of the misfolded conformation may lead to a better understanding of protein folding diseases. The mechanism of protein folding for a multidomain dimer is complex and the interactions that stabilize dimeric proteins are derived from intersubunit and intrasubunit interactions. The focus of this study is to monitor the unfolding and dissociation of homodimeric bovine plasma amine oxidase (BPAO) using the chemical denaturants: guanidine hydrochloride (GuHCl), urea and sodium dodecyl sulfate (SDS).

BPAO has a molecular weight of about 192kD. Each subunit contains a copper and 2, 4, 5-trihydroxyphenylalanine quinone (TPQ) cofactors at the active site that aid in the oxidative deamination of biogenic amines to the corresponding aldehydes. The x-ray crystal structure shows that each active site is composed of residues from both monomers and there are two arms that extend from one monomer to the other that aid in the intermolecular interaction.

Each denaturant interacts with BPAO differently and therefore, the pathways for denaturation are different. Spectroscopic, chromatographic and electrophoretic techniques were used to study the denaturants interactions with BPAO and different pathways of denaturation have been proposed. The addition of SDS leads to the dissociation of the homodimer and then unfolding of the monomers, with a 12.7Kcal/mol free energy of denaturation. Urea interacts with the dimer by unfolding it into several dimeric intermediates that are not molten globule in character, and at high concentrations, it promotes dissociation. The free energy of denaturation calculated is 4.75Kcal/mol. GuHCl interacts with BPAO by the formation of a molten globule dimer, which then undergoes dissociation to form a molten globule monomeric intermediate. The free energy of denaturation in GuHCl is 15.3Kcal/mol, which is similar to that in SDS but much higher than in urea. Therefore, final conformation of the protein in GuHCl and SDS are similarly denatured, while BPAO in urea is only partially unfolded.

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List of Abbreviations

ΔASA	change in accessible surface area
ΔC_p	change in heat capacity
$\Delta G_{H_2O}^0$	free energy for the denaturation transition in the absence of denaturant
ΔG	free energy
ΔG_D	free energy of dissociation in the absence of denaturant
ΔG_{total}	total free energy of unfolding and/or dissociation in the absence of denaturant
ΔG_U	free energy of unfolding in the absence of denaturant
ΔG_U^D	free energy of unfolding of the dimer in the absence of denaturant
ΔG_U^M	free energy of unfolding of the monomer in the absence of denaturant
ΔH_D	enthalpy at the melting temperature
ΔN	change in refractive index
Γ	emissive radiative rate from excited state
α	pre-exponential factor
β	partial anisotropy
ε	extinction coefficient
ϕ	rotational correlation time
λ_{max}	wavelength with maximum emission intensity
τ	lifetime
θ	fraction of ligand bound
$\bar{v}$	number of ligands bound per enzyme
$[L]_0$	total ligand concentration
$[L]_{free}$	free ligand concentration
$\langle r \rangle$	emission anisotropy
$\partial \Delta G$	change in free energy of denaturation
AGAO	<i>Arthrobacter globiformis</i> amine oxidase
ANS	1-anilinonaphthalene-8-sulfonic acid; 1,8-ANS
APS	ammonium persulfate
BCA	bichinchinoic Assay
Bis-ANS	4,4'-dianilino-1,1'-binaphthyl-5,5'- disulfonic acid, dipotassium salt
BPAO	bovine plasma amine oxidase
BSA	bovine serum albumin
CAO	copper amine oxidase
Cm	denaturant concentration at the midpoint of a sigmoidal transition
CSM	center of spectral mass
D	denaturant
D(t)	difference function
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
E	enzyme
ECAO	<i>Escherichia coli</i> amine oxidase

F	fluorescence emission intensity
f	friction coefficient
F_0	fluorescence intensities in the absence of quencher
f_a	fraction of the initial fluorescence that is accessible to the quencher
FAD	flavin adenine dinucleotide
$F_{\max}$	maximum fluorescence intensity
FRET	Förster resonance energy transfer
f_U	fraction of protein unfolded or dissociated
GuHCl	guanidine hydrochloride
HPAO	<i>Hansenula polymorpha</i> amine oxidase
HPLC	high performance liquid chromatography
I	inhibitor
II	integrated intensity
K_A	association constant; $1/K_D$
K_{av}	distribution constant
K_D	dissociation constant; $1/K_A$
K_i	inhibition constant
K_m	Michaelis constant; substrate concentration at $\frac{1}{2} V_{\max}$
k_q	bimolecular quenching constant
K_Q	quenching constant for the exposed fluorophores
K_{SV}	Stern-Volmer quenching constant
K_U	equilibrium constant
L	ligand
LSAO	lentil seedling amine oxidase
LTQ	lysine tyrosylquinone
M	molecular weight
m_U^D	m values for dimer unfolding
m_U^M	m values for monomer unfolding
MAO	monoamine oxidase
m_D	m values for dimer dissociation
m_{total}	total m value
n	number of binding sites
N	number of fluorophores in the excited state
P	protein concentration
PAGE	polyacrylamide gel electrophoresis
PM	N-(1-pyrene)maleimide; pyrene maleimide
PPAO	pig plasma amine oxidase
P_t	protein concentration in monomers
q	net charge of protein
R	gas constant ($1.987 \text{ cal.M}^{-1}.\text{K}^{-1}$ or $8.314 \text{ J.M}^{-1}.\text{K}^{-1}$)
$r(t)$	decay of emission anisotropy
R_0	Förster distance; distance for 50% energy transfer between two fluorophores
r_0	limiting anisotropy
r^2	correlation coefficient
R_f	column flow rate

R_s	Stokes radius
R_{sp}	specific resolution
R_t	retention time
Rubisco	ribulose biphosphate carboxylase/oxygenase
S	substrate
$S(t)$	sum function or total emission intensity
SA	specific activity
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SSAO	semicarbazide-sensitive amine oxidase
T	temperature
TEMED	N,N,N',N'-tetra-methylethylenediamine
T_m	melting temperature
TPQ	2,4,5-trihydroxyphenylalanine quinone
TR	time resolved
TREA	time resolved emission anisotropy
u	mobility
UGGE	urea gradient gel electrophoresis
v	velocity
V_0	void volume
V_e	elution volume
V_{max}	maximum velocity
V_p	peak volume
V_t	total volume
y_D	intercept of the denatured state
y_N	intercept of the native state

Chapter 1: Introduction

1.1 The Relevance of Protein Folding

The improper folding of proteins has been implicated in several disease states, including α -synuclein in Parkinson's Disease [1], β -amyloid in Alzheimer's Disease, huntingtin in Huntington's Disease, and prion proteins in prion diseases [2]. A more detailed understanding of the pathways leading to improperly folded proteins may lead to further understanding of protein-folding diseases. Such information may also lead to the development of therapeutics that prevent the accumulation of non-native protein species that lead to disease, for example by inhibiting the formation pathway for misfolded proteins.

Numerous studies have been undertaken to elucidate the mechanisms by which a protein folds to form a three-dimensional structure capable of supporting a particular function. Many of the studies on protein folding have been accomplished with small single (ubiquitin, cytochrome c [3]) or multidomain proteins (α -lactalbumin, lysozyme and aldolase [4]). Less information is known about more complex proteins with multiple domains and/or subunits, where the folding may be governed by both monomer folding and subunit association. By characterizing the intramolecular and intermolecular interactions of the amino acid sequence, further information on the conformational properties and folding pathways of proteins may be determined.

In order to understand the manner by which proteins fold, *in vitro* techniques measure the conversion of either the native state to the unfolded one, or *vice versa*. The native state of a protein is the one which has a specific three-dimensional structure that is

necessary for a particular function. In contrast, the unfolded state is usually inactive because the structure has been compromised. Often, the folding of a protein is not simply the transition from an unfolded to a specific active folded conformation, but rather proceeds through intermediate states that possess various degrees of structure and function. One such intermediate is the molten globule, which has native-like secondary structure but lacks a rigid tertiary structure (Section 1.2.1).

In vitro, the protein undergoes a transformation from one state to another with perturbants which include temperature, pressure or various chemical denaturants. The increase or decrease in temperature or pressure can result in dramatic changes in protein conformation, which can stabilize or destabilize the structure. Similar results are seen with the chemical perturbants, usually guanidine hydrochloride (GuHCl), urea and sodium dodecyl sulfate (SDS).

1.1.1 Aims of the thesis

The aim of this thesis is to investigate the folding and refolding pathways for a stable homodimeric metalloenzyme, bovine plasma amine oxidase (BPAO, 192 kD) as an example of a high molecular weight, multisubunit protein. It is anticipated that these studies will provide insights into the forces that control specific protein folding pathways and subunit association. This thesis will focus specifically on:

1. Monitoring the global and local changes in BPAO structure during unfolding and dissociation.
2. Detecting the presence of intermediates during perturbation by denaturants.

3. Determining the pathways for unfolding and/or dissociation.
4. Calculating the thermodynamics for a specified pathway.

1.2 The Protein Folding Problem

Proteins can assume a large number of conformations. According to Levinthal's Paradox [5, 6], a protein of 150 residues is expected to have 3^{300} (10^{150}) possible conformations, assuming that each amino acid can have 3 possible conformations around the C_α atom (C_α -N (ϕ) and C_α -C (ψ)). If the protein can explore new conformations at a rate of 10^{13} per second, then it will take the protein 10^{130} years (or 10^{137} seconds) to explore all conformations. However, the proteins refold during a finite timescale (minutes or hours) and adopt a particular conformation, driven by free energy and entropy considerations. In the energy landscape of a protein, it is theorized that there are microstates with lower free energies than the native state [7]. Therefore, the native state of a protein is believed to be a collection of several slightly different microstates, and could be in either a global or local minimum in the free energy [7].

When the polypeptide is on the ribosome or completed and released from the ribosome, the protein can adopt a native conformation either in the absence or presence of molecular chaperones [8]. It is difficult to simulate *in vitro* the folding of a particular amino acid sequence to the lowest energy state [9].

Generally, for soluble proteins, most charged groups are on the protein surface, while nonpolar groups form hydrophobic interactions within the protein [10]. Stabilization of globular proteins is typically 2 – 42 kcal/mol [10, 11] (Tables 1, 2 and 3).

In addition, the protein has to exhibit flexibility for enzymatic activity. Therefore, there is a delicate balance between flexibility and the interactions that stabilize the protein [12].

1.2.1 Intermediate States

The transition from a native protein conformation to an unfolded state is not necessarily a simple two-state transition from a folded to an unfolded conformation. Rather, it is possible that stable intermediate conformations such as the molten globule exist, leading to complex multi-state transitions.

The molten globule intermediate has conformational characteristics of both the native and unfolded protein and is commonly found in protein folding pathways [13-18]. It has a high degree of native-like secondary structure, but little or no tertiary structure [14, 18, 19]. So, side chain interactions that stabilize the native state of the proteins are decreased. It can be thought of as being a nonspecific assembly of secondary structure segments [15] whereby hydrophobic areas within the protein structure in the native state are exposed [16]. The molten globule is present as an equilibrium intermediate or a transient intermediate that is formed early in the refolding of a protein from the denatured state [13, 17]. This molten globule conformation is induced by changes in temperature, pH or with the addition of chemicals such as urea and guanidine hydrochloride [16, 20-23].

1.2.2 Assembly of multi-subunit structures

An increase in the complexity of the protein folding pathway is expected with increasing numbers of subunits. The unfolding pathway for a dimeric protein (N_2) can

proceed to either an unfolded dimeric (N_2^U) or an unfolded monomeric state ($2M^U$). If there are strong interfacial interactions between the monomers, the unfolded dimer will be favored prior to dissociation (e.g. Sec A [24]) whilst stronger intramolecular interactions within monomers will favor dissociation with subsequent unfolding of the monomer (e.g. desulfoferrodoxin [25]).

1.3 Conformational Stability of a Protein

The protein folding pathway may depend on the nature of the perturbant: chemical denaturation routes can be different from those promoted by thermal or hydrostatic pressure perturbation. Furthermore, the choice of denaturant (such as guanidine hydrochloride, urea, SDS or increased salt concentration) may also influence the unfolding pathway due to differences in their interactions with the protein.

Solvents interact with proteins in two different ways [26]. There is the surface protein/solvent contact and the permeation of the protein by solvent that interacts with the interior. In the transition state, an intermediate is formed as a result of the solvent permeation that causes swelling of the protein [26].

Tanford [27] proposed stoichiometric binding and Schellman [28] suggested a thermodynamic approach to denaturants interacting with proteins. The free energy of denaturation is the sum of the free energy contributions from all exposed protein moieties [28]. It is assumed that in addition to direct interactions of the denaturant *via* binding, indirect interactions *via* long-range forces or through intermediate molecules is possible [28].

1.3.1 Mechanism of Solvent Denaturation

Solvent denaturants used routinely to perturb protein structure for conformational studies include chaotropic chemicals such as cationic GuHCl, neutral urea and anionic SDS, shown in Figure 1, below.

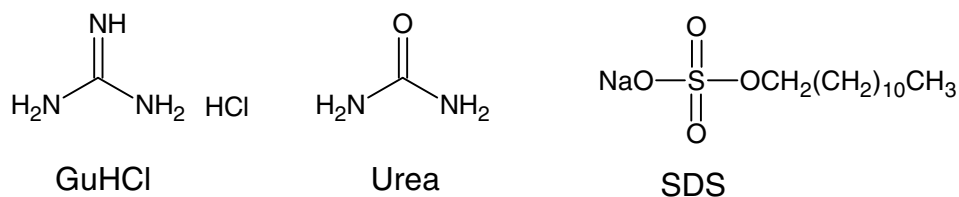


Figure 1: Denaturants used to monitor protein conformation: guanidine hydrochloride (GuHCl), urea and sodium dodecyl sulfate (SDS).

The mechanisms of denaturation for different denaturants vary. SDS is routinely used for the size separations of proteins by gel electrophoresis. The maximum binding ratio is 1.4g SDS per g protein [29]. SDS leads to the dissociation of oligomeric proteins [30, 31]. It can form micelles above a critical micelle concentration (cmc). At SDS concentrations lower than the cmc, monomeric SDS binds to proteins [27, 32, 33], while at higher concentrations, it forms micelles that denature proteins by interactions of the sulfate with the positively charged amino acid side chains (electrostatic) and the alkyl chain with hydrophobic side chains (hydrophobic) of proteins [33, 34]. There are two models for the interaction of SDS micelles with proteins: protein decorated micelle structure [35] and necklace structure [36, 37]. According to the protein decorated micelle model, the hydrophilic head of SDS molecules interact with the polypeptide chain by

hydrogen bonds [35, 38] on the surface of a large SDS micelle. In the necklace model, multiple detergent micelles form along the polypeptide chain [36].

Information about the mechanism of denaturation by GuHCl and urea is rather limited and not well understood. GuHCl binds to ~4 amino acids in proteins and the number of guanidinium ions associated with the protein increases with protein unfolding [39]. It interacts with both the side chains and the protein backbone through hydrogen bonds [40, 41]. Since the concentration of GuHCl used in protein studies is typically 1-5M and the concentration of protein is <1 μ M [40], then the effect of the charge from the denaturant on the protein is predominant [42]. Specifically, the introduction of a large positive charge into the protein solution neutralizes the stabilizing effect of electronegative interactions and leads to the denaturation of the protein [42]. Therefore, the effect of GuHCl on proteins is actually reflecting the contributions of hydrophobic interactions [42].

Urea is an uncharged destabilizing solute. One urea molecule interacts with 3-4 amino acid residues through hydrogen bonds [43, 44]. It interacts with the protein not only at the surface but also in the hydrophobic interior [45, 46] and interacts with both polar and nonpolar protein groups [45]. Since urea does not have an ionic effect, the measured stability of a protein is based on hydrophobic and electrostatic interactions [42]. The denaturation effects of urea are also interpreted as an effect on solvent: it makes the solvent more nonpolar by solvating the polypeptide chain better than water [45].

The difference in the mechanism of interaction of GuHCl and urea with a protein is clearly seen in the transition midpoints (C_m) of equilibrium two-state denaturation experiments. The C_m ratio of urea/GuHCl ranges from 2 to 3.66 [47, 48]. Thus, a higher

concentration of urea is necessary to exhibit the equivalent protein conformational change induced by GuHCl.

1.3.2 Protein Folding Mechanisms

For small molecular weight proteins, in the hierarchy of protein folding, secondary structure, domains and subunits form by a sequential mechanism [10]. The energy landscape is smooth [49], which suggests the presence of a single low energy conformation. In contrast, larger molecular weight proteins fold by a parallel mechanism [49]. According to this mechanism, the protein can achieve the native conformation through multiple pathways. Therefore, the energy landscape of the native protein is expected to be rough, with many transient intermediates [49].

For some small, single domain proteins, the folding pathway is known [3, 4]. Examples include ubiquitin and cytochrome c [3]. By characterizing the intra- and intermolecular interactions of the amino acid sequence, further information on the conformational properties and folding pathways of other proteins may be determined. However, for more complicated large molecular weight proteins with multiple domains/subunits where folding is governed by both monomer folding and subunit association, less information is known [4].

Numerous studies have been undertaken to elucidate folding pathways. In addition to the simple two-state transition mentioned above ($N \rightarrow U$), the transition from the native (N) to denatured state (U) can involve three-states or more, with observable protein folding intermediates (I) such as the molten globule (MG). Thus, for simple

monomeric proteins, the transition $N \rightarrow U$ or $N \rightarrow I \rightarrow U$ is expected [20, 50]. The number of intermediates may vary.

Oligomeric proteins undergo a more complex denaturation process. In addition to unfolding of the polypeptide as in the monomeric protein (M), there is the dissociation of subunits. Thus, for a dimer, the two mechanisms for a simple two-state transition for denaturation involve dimer unfolding ($N_2 \rightarrow N_2^U$) or dissociation ($N_2 \rightarrow 2M^U$). For a three-state process, dimer unfolding can occur by dimeric intermediates only ($N_2 \rightarrow N_2^A \rightarrow N_2^U$) and dissociation can occur by a dimeric ($N_2 \rightarrow I_2 \rightarrow 2M^U$) or monomeric intermediate ($N_2 \rightarrow 2M \rightarrow 2M^U$).

The simplest two state model for a tetramer is dissociation into four monomers, $N_4 \rightarrow 4M^U$ [51-53] or into 2 dimers, $N_4 \rightarrow 2N_2$ [54]. Furthermore, the mechanism of denaturation can be a three-state process with a tetrameric ($N_4 \rightarrow N_4^{\text{inactive}} \rightarrow 4M^U$) [55], dimeric or monomeric intermediate.

1.3.3 Monitoring Stability of a Protein

The conformational stability of a protein is measured by the free energy of denaturation (ΔG), which compares the denatured conformation relative to the native one. The structure of a protein can be perturbed as stated above by temperature [56, 57], pressure [58, 59], pH [60, 61] and with the addition of solvent denaturants like glycerol, guanidine hydrochloride (GuHCl), urea or sodium dodecyl sulfate (SDS). The use of hydrostatic pressure and temperature to perturb proteins allows measurement of structural changes without the addition of extrinsic factors to the solution.

Thermal denaturation is used to monitor the free energy of the protein (ΔG) by Equation 1 [57, 62]:

$$\Delta G = \Delta H_D \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left[(T_m - T) + T \ln \left(\frac{T}{T_m} \right) \right] \quad (1)$$

where ΔH_D is the enthalpy at the melting temperature, T_m , and ΔC_p is the change in heat capacity.

Pressure and solvent denaturant studies use the fraction of perturbed protein (K_U) to calculate the free energy [63]. Only solvent perturbation is used in this study. The two methods used to calculate the free energy of denaturation are linear-extrapolation and non-linear least squared analysis. For the linear extrapolation method, the assumptions are that [64]:

- 1) The transition of denaturation reflects a two-state transition from the native (N) to denatured (U) conformations; and
- 2) The denaturation and renaturation are reversible.

The free energy is analyzed by the linear extrapolation method in zero concentration of denaturant [65, 66] as described below.

The equilibrium constant (K_U) for unfolding is given by:



$$K_U = \frac{[U]}{[N]} \quad (2)$$

and the free energy of denaturation (ΔG) is calculated by Equation 3:

$$\Delta G = -RT \ln K_U \quad (3)$$

where [U] and [N] are the respective concentrations of the unfolded and native state, R is the gas constant and T is the absolute temperature. The free energy of denaturation can also be calculated from the free energy in the absence of denaturant ($\Delta G_{H_2O}^0$), the slope of free energy as a function of denaturant concentration (m), and the denaturant concentration (D) in the transition region by the equation:

$$\Delta G = \Delta G_{H_2O}^0 - m[D] \quad (4)$$

The $\Delta G_{H_2O}^0$ is an intrinsic property of the protein rather than the denaturing process [66, 67]. The transition mid-point concentration, C_m , is calculated from Equation 5:

$$C_m = \frac{\Delta G_{H_2O}^0}{m} \quad (5)$$

The above equations are valid for calculating unfolding parameters for monomers and oligomers. However, for dissociation of a dimer into monomers, the dissociation constant (K_D) is:

$$N \rightarrow 2U \quad (6)$$

$$K_D = \frac{[U]^2}{[N]} = 2P_t \left[\frac{f_U^2}{1 - f_U} \right] \quad (7)$$

where f_U is the measured fraction dissociated and P_t is the protein concentration in monomers. The ΔG is determined from the dissociation constant (K_D), by Equation 8:

$$\Delta G = -RT \ln K_D \quad (8)$$

and the denaturant concentration at the transition mid-point for dissociation, C_m , is calculated [68]:

$$C_m = \frac{RT \ln[P_t] + \Delta G_{H_2O}^0}{m} \quad (9)$$

In addition to the linear extrapolation model for denaturation, the non-linear least squared analysis models may also be used (Equations 10, 11 and 12). It has the benefit of adding pre- and post-transition baselines to the two-state transition from the native to the denatured states. This gives a more accurate estimation of the free energy of denaturation and m value if the native (pre-transition baseline) and denatured (post-transition baseline) protein conformations contribute to the transition free energy. For unfolding, where y is the measured parameter, a simple two-state transition [64] is defined as:

$$y = \frac{y_N + y_D e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}}}{1 + e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}}} \quad (10)$$

or with pre- and post-transition baselines [65]:

$$y = \frac{\left[(y_N + m_N[D]) + (y_D + m_D[D]) e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}} \right]}{1 + e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}}} \quad (11)$$

where y_N and y_D are the y intercepts for the native and denatured states, m_N and m_D are the slopes of the pre- and post-denaturation region, $\Delta G_{H_2O}^0$ is the free energy for the denaturation transition in the absence of denaturant and m is the slope of free energy in the denaturation transition.

For the two-state dissociation of a dimer, the non-linear equation [68] is:

$$y = y_N - (y_N - y_D) \frac{\left(\sqrt{\frac{1 + 8P_T}{e^{\frac{-(\Delta G_{H_2O}^0 - m[D])}{RT}}}} - 1 \right)}{4P_T} e^{\frac{-(\Delta G_{H_2O}^0 - m[D])}{RT}} \quad (12)$$

The m value is a measure of the ability of a denaturant to perturb a protein and is determined from the change in free energy of denaturation ($\partial\Delta G$) with a change in denaturant concentration ($\partial[D]$) (from Equation 4):

$$m = \frac{\partial \Delta G}{\partial [D]} \quad (13)$$

Myers [69] compared 45 proteins with a maximum of 415 residues and related the m value to a change in accessible surface area (ΔASA) upon perturbation by GuHCl and urea.

1.3.4 Free energy of protein denaturation

Quaternary interaction is an additional element of stabilization inherent to oligomeric proteins [70-72]. It can provide as much as 65-100% of the conformational stability of dimers [72] with dissociation free energies (ΔG_D) as high as 36 kcal/mol [73]. This is greater than the unfolding free energy (ΔG_U) of monomeric proteins in either GuHCl or urea, which vary from 3 – 9 kcal/mol (Table 1).

The thermodynamics of denaturation for several dimeric proteins are summarized in Tables 2 and 3. A comparison of the free energy for dimer unfolding, dissociation and monomers unfolding is shown in Table 2. For some proteins, the dimeric state is very stable and undergoes dimeric unfolding prior to dissociation (e.g. ascorbate oxidase [74]) while for others, it is only marginally stable and tend to dissociate without dimeric unfolding (e.g. creatine kinase [75]). Procaspase-3 [76] shows the presence of both stable dimers and stable monomers. With the exception of the lowest (creatine kinase [75]) and highest (organophosphorus hydroxylase [73]) ΔG_D , the range is 9 to 17 kcal/mol for dissociation. With the exception of the highly stable dissociated monomers which are 14 -

17 kcal/mol, the ΔG_U for both dimers and dissociated monomers are generally 3 - 8 kcal/mol which is comparable to monomeric unfolding in Table 1.

Table 3 shows the total free energy (ΔG_{total}) and m values for different dimeric denaturation pathways: two-state dissociation, three-state dissociation with dimeric or monomeric intermediate and a four-state transition with both monomeric and dimeric intermediates. There are no specific trends when comparing one pathway to another or the effect of molecular weight. In general, the ΔG_{total} is between 9 to 26 kcal/mol for dimers but there are a few more highly stable dimers with free energies of 33 – 42 kcal/mol.

Table 1
Thermodynamic parameters for monomeric protein unfolding
by urea and GuHCl at 25°C.

Protein	Conditions	D ⁴	¹ ΔG_U (kcal/mol)	² m (kcal. mol ⁻¹ .M ⁻¹)	³ C _m (M)	Ref
Cytochrome-c	pH 6, 0.1-1M KCl	GuHCl	8.18±0.42	3.31±0.17	2.47	[77]
	pH 6, 0.1-1M KCl	Urea	8.02±0.43	1.28±0.07	6.26	
Interleukin-6	10mM Tris-HCl, pH 7.4	GuHCl	1.8±0.3	1.0±0.2	1.7	[78]
	10mM Tris-HCl, pH 7.4	Urea	3.4±0.6	0.7±0.1	4.8	
Lysozyme	pH 2, 0.1-1M KCl	GuHCl	4.18±0.21	2.03±0.10	2.06	[77]
	pH 2, 0.1M KCl	Urea	4.09±0.26	1.23±0.08	3.32	
RNase-A	pH 3, 0.1-1M KCl	GuHCl	5.03±0.21	2.83±0.12	1.78	[77]
	pH 3, 0.1M KCl	Urea	4.07±0.16	1.98±0.08	2.05	
Thioredoxin	0.05M MOPS pH 7	GuHCl	7.8±0.2	3.51	2.22	[66]
	0.05M MOPS, 0.5M NaCl pH 7	Urea	8.6±0.9	1.32	6.57	

¹ ΔG_u represents the free energy of monomeric unfolding (Equation 4).

²m represents the slope of the free energy as a function of denaturant (Equation 13).

³C_m represents the transition mid-point and is measured in M (Equation 5).

⁴D is denaturant

Table 2
Conformational stability of the unfolding and dissociation of dimeric proteins

Protein	¹ ΔG_U (kcal/mol)	² ΔG_D (kcal/mol)	References
Ascorbate oxidase	3.5 ^d	12 - 13	[74]
Creatine kinase	2.61 ^m	3.92	[75]
Gluthatione transferaseM1-1	16.50±0.13 ^m	10.81±1.8	[79]
Methionine adenosyltransferase III	3.53±0.39 ^m	8.64±0.55	[71]
Organophosphorus hydrolase	4.32±0.26 ^d	36.1±1.9	[73]
Procaspase-3	8.3±1.3 ^d 7.0±0.5 ^m	10.5±1.0	[76]
Prostatic acid phosphatase	3.38 ^m	4.24	[80]
SecA	8.4±1.6 ^d	14.1±1.1	[24]
Triosephosphate isomerase	3.97±0.17 ^m	16.8±0.26	[81]
Tyrosyl-tRNA synthetase	13.9±0.6 ^m	13.8±0.2	[82]

^m unfolding of the monomer

^d unfolding of the dimer

¹ unfolding of the monomer (m) and dimer (d) determined by Equations 2, 3 and 4.

² dissociation of the dimer determined by Equations 7, 8 and 4.

Table 3
Thermodynamic parameters for the equilibrium denaturation of dimers

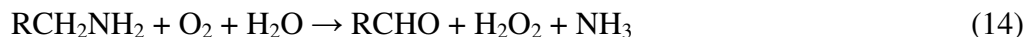
Protein	MW (kD)	Denaturant	^a ΔG_{total}	^b m_{total}	Reference
<i>N₂ ↔ 2U</i> <i>Apparent two-state process</i>					
Agglutinin I	25	GuHCl	13.13		[83]
Arc repressor	14	GuHCl	11.1	3.27	[84]
		Urea	11	1.91	
Archaeal histones	16		14.3		[85]
C _H 3 domain	24		15.8		[86]
Glutathione S-transferase	50	Urea	26.0±1.7	4.5	[87]
Human interferon gamma	34		7.2 – 8.4		[88]
<i>N₂ ↔ I₂ ↔ 2U</i> <i>Dimeric intermediate</i>					
Bacterial luciferase	76	Urea	24		[80]
Organophosphorus hydrolase	72	Urea, GuHCl	40.42		[73]
SecA	204	Urea	22.5	5.6	[24]
Tyrosyl-tRNA synthetase		Urea	41.6	5.9	[82]
<i>N₂ ↔ 2I ↔ 2U</i> <i>Monomeric Intermediate</i>					
		GuHCl	15.4	4.7	[74]
Ascorbate oxidase	140	Urea	17.2	2.9	[74]
Desulfoferrodoxin	28	GuHCl	34.7		[89]
Glutathione transferase-1	50	GuHCl, Urea	21.6		[79]
Methionine adenosyltransferase III		Urea	15.7	7.25	[71]
Prostatic acid phosphatase	100	GuHCl	11		[80]
Triosephosphate isomerase		GuHCl, Urea	24.7		[81]
Tryptophan repressor		Urea	22.4±0.7	2.8	[90]
<i>N₂ ↔ I₂ ↔ 2I ↔ 2U</i>					
Creatine kinase	86	Urea, GuHCl	9	11.5	[80]
Ketosteroid isomerase		Urea	22.0	4.0	[91]
Procaspase-3	63	Urea	32.8	5.7	[76]

^a kcal/mol^b kcal.mol⁻¹.M⁻¹

1.4 PROTEIN UNDER STUDY

1.4.1 Bovine Plasma Amine Oxidase

BPAO catalyzes the oxidative deamination of amines into their corresponding aldehydes according to Equation 14:



There are two of classes of amine oxidases which are distinguished by their different cofactors [92, 93]. Monoamine oxidase (MAO), a membrane associated protein, contains a flavin adenine dinucleotide (FAD) cofactor. The second class is the copper amine oxidases that are quinoproteins. In addition to a copper (II) ion, copper amine oxidases also have lysine tyrosylquinone (LTQ) or 2, 4, 5-trihydroxyphenylalanine quinone (TPQ) cofactors (Figure 2) [92]. Thus far, the only protein identified with LTQ is lysyl oxidase [94, 95]. BPAO is a member of the copper amine oxidase family and has a TPQ cofactor [96].

Other copper amine oxidases (CAOs) with TPQ include mammalian diamine oxidase (porcine kidney, human kidney and human placenta), plant diamine oxidase (lentil seedling, pea seedling, maize, peanut, soyabean, fenugreek), microbial amine oxidase (*Aspergillus niger*, *Hansenula polymorpha*, *Escherichia coli*, *Klebsiella aerogenes*, *Arthrobacter globiformis*) and mammalian plasma amine oxidase (bovine and porcine) [97]. Copper amine oxidases are ubiquitous homodimeric proteins that are found in most living organisms: bacteria, plants and mammals [92, 93, 97-99].

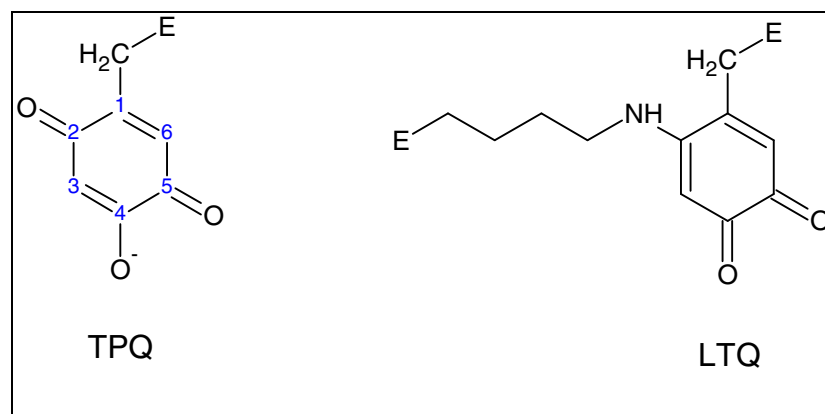


Figure 2: Prosthetic groups of copper amine oxidases: TPQ (2, 4, 5-trihydroxyphenylalanine quinone) and LTQ (lysyl tyrosyl quinone).

1.4.1.1 Physiological Significance

In microorganisms, copper amine oxidases (CAOs) function in nutrient catabolism and enable amine substrates to be used as a source of carbon and nitrogen. Their roles in higher organisms are more complex and are linked to the metabolism of biogenic amines [98]. In plants, they catalyze cell-wall crosslinking, lignification and defense, while in mammals, tissue specific forms are possibly involved in cell signaling (polyamines [100] and H_2O_2 [101]), growth and development (cross linking of collagen and elastin), protein and cell regulation [100, 102] and programmed cell death by the regulation of intracellular polyamines [103]. In addition, copper amine oxidases are medically relevant [104]. Recently, a plasma amine oxidase (known as vascular adhesion protein 1 (VAP-1)) has been found to mediate interactions between lymphocytes and endothelial cells, contributing to the formation of vascular plaques implicated in congestive heart failure, causing vascular damage, and an increase in the risk of cancer in women [105].

1.4.1.2 Cofactors

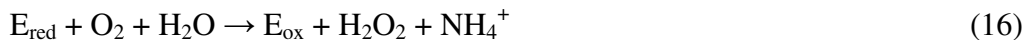
There are two cofactors in BPAO: copper and TPQ. Both are essential for activity. The role of the copper cofactor in BPAO has been elucidated by metal substitution studies [106-112]. Substitution of the copper (II) ion with cobalt, nickel or zinc resulted in a loss of activity [107]. Half and fully depleted copper sites resulted in 82% and total loss in activity, respectively [110] but activity can be recovered with the addition of Cu^{2+} [108]. The metal binding site coordinated by three invariant histidine residues (H519, H521 and H683) [113]. While copper has no catalytic function, it is

necessary to maintain the structure of the active site [111, 114]. It is also necessary for TPQ biogenesis [115, 116] and has a possible role in the oxidative half reaction (Equation 16) [117-120] but not the reductive half reaction (Equation 15) [99, 117, 121, 122] for BPAO enzyme catalysis.

BPAO was the first CAO in which the new redox cofactor, 2, 4, 5-trihydroxyphenylalanine quinone (TPQ), was identified [96]. TPQ is essential for the functioning of BPAO. It is derived from a post-translationally modified tyrosine (Y470) residue [116, 123] found in the consensus sequence NYD/E [89]. It is generated by a self-processing mechanism whereby the active site copper catalyses a dioxygen-dependent insertion of oxygen into the tyrosine ring and the addition of water or hydroxide ion leads to TPQ formation [93]. A detailed mechanism for the biosynthesis of TPQ is proposed based on X-ray crystal structures [115, 116].

1.4.1.2.1 Catalytic Function

The oxidative deamination of amines by BPAO occurs at the primary amino termini of the substrate [124] by a ping-pong mechanism that consists of two half reactions (Equations 15 and 16) [92] summarized in Equation 14:



The reductive half-reaction (Equation 15) consists of the abstraction of the amine proton by an active site base and the formation of a Schiff base complex between the

substrate and TPQ followed by the release of the aldehyde product [199, 117, 121, 122]. The modified TPQ cofactor is regenerated with the addition of oxygen by the oxidative half-reaction (Equation 16), which involves the copper (II) ion [92, 117-120].

1.4.1.3 cDNA & primary structure

The full-length cDNA of BPAO has been isolated from a bovine liver cDNA library [123]. Translation allows comparison of the 762 residue protein (Figure 3) to that of other copper amine oxidases. The length of the primary sequences for copper amine oxidase monomers varies from 587 for lentil seedling amine oxidase (LSAO) to 762 amino acids for BPAO [97], and homology is in the range of 15 - 91% (Figure 4). There are 33 conserved residues and high sequence homology at the C-terminal tail region [124].

The BPAO dimer has a molecular weight of 170 kD from the amino acid sequence (Figure 3) [123]. There are 30 tryptophan, 62 tyrosine, 92 phenylalanine and 20 cysteine residues per dimer. At the N-terminal region of each monomer, there is a hydrophobic 16 amino acid sequence that shows features of a possible signal peptide [123]. This is consistent with the biosynthetic pathway for BPAO, which is an extracellular protein that is produced in the liver [123]. Additionally, there are three N-linked glycosylation sites at ¹³⁶NVT, ²³¹NIT and ⁶⁶⁵NET in each monomer [113]. The isoelectric point (pI) is 5.78 [125].

mfififlslw tllvmgreeg gvgseegvgk qchpslpprc psrspsdqpw thpdqsqifa	60
dlsreelttv msfltqqlgp dlvdaaqarp sdncvsvsel qlppkaaala hldrgspppa	120
realaivffg gqpqpntel vvgplpqpsy mrdvtverhg gplpyyrrpv llreyldidq	180
mifnrelpqa agvlhh <u>cc</u> sy kqggqklltm nsaprgvqsg drstwfgiyy nitkggpylh	240
pvglellvdh kaldpadwtv qkvffqgryy enlaqleeqf eagqvnvvi pddgtggfws	300
lksqvppgpt pplqfhpqgp rfsvqgnrva sslwtfsfgl gafsgprvfd vrfqgerlay	360
eislqeagav yggntpaaml trymdsgfgm gyfatplirg vd <u>cp</u> ylatym dwhfvvesqt	420
pkthdaf <u>cy</u> feqnkgplr rhhsdflshy fggvaqtlv frsvstml <u>ny</u> dyvwdmvfyp	480
ngaievkha tgyissalf gaarrygnqv gehtlgpvht hsahykvldd vgglenwwa	540
edmafvtai pwspehqiqr lqvtrkqlet eeqaafplgg aspnylylas kqsnkwghpr	600
gyriqtvsfa ggpmpqnspm erafswgryq laitqrkete psssvfnqn dpwtptvdfs	660
dfinnetiag kdlvawvtg flhiphaedi pntvtvgngv gflrpydff dqepsmdsad	720
siyfregqda gsceinplac lpqaatcapd lpvfshggyp ey	762

Figure 3: Amino acid sequence of BPAO. The consensus sequence for TPQ biogenesis is shown in bold (**nyd**) and the underlined cysteine residues show the intrasubunit disulfide bonds: C197-C198 and C403-C429. The bold cysteine (C94) is the only surface sulfhydryl residue. The numbers of the last residue is shown on the right [123]. (Accession number A54411)

ECOLI	100										
AMO_KLEAE	83.88	100									
AOC2_HUMAN	17.33	17.06	100								
AOC3_HUMAN	15.98	15.83	64.42	100							
AOC3_MOUSE	15.19	13.32	62.43	83.09	100						
BSAO - liver	14.53	12.93	60.58	79.53	76.25	100					
BSAO - lung	15.32	14.91	60.19	80.84	78.08	91.21	100				
AGAO	23.35	24.14	16.46	18.03	15.20	16.93	15.83	100			
AG_HAO	24.89	25.33	16.84	17.28	16.69	17.28	16.11	59.56	100		
LSAO	22.82	22.52	14.71	14.71	15.47	15.92	14.57	20.22	21.47	100	
PSAO	23.59	22.85	14.99	14.99	14.54	15.13	14.99	20.69	22.40	90.99	100
	ECOLI	AMO_KLEAE	AOC2_HUMAN	AOC3_HUMAN	AOC3_MOUSE	BSAO-liver	BSAO-lung	AGAO-PEAO	AGAO-HAO	LSAO	PSAO

Figure 4: Sequence homologies (in %) of amine oxidases from various sources. The sequences were aligned using the ClustalW 1.81 program for multiple sequence alignment [126]. The input sequences for the CAO from the SWISS-PROT database with the accession number in parentheses are: ECAO (P46883), AMO-KLEAE (*Klebsiella aerogenes* amine oxidase) (P49250), AOC2-HUMAN (Human retina-specific copper amine oxidase precursor) (O75106), AOC3-HUMAN (Human membrane copper amine oxidase, vascular adhesion protein-1) (VAP-1) (Q16853), AOC3-MOUSE (mouse vascular adhesion protein-1) (O70423), BSAO-liver (Q29437), BSAO-lung (O46406), AGAO-PEAO (phenylethylamine oxidase from *Arthrobacter globiformis*) (P46881), AGAO-HAO (histamine oxidase from *Arthrobacter globiformis*) (Q59118), LSAO (P49252) and PSAO (Q43077).

1.4.1.4 Enzyme Structure

The X-ray crystal structure of BPAO was recently determined (Figure 5) [113, 127] and is quite similar to the X-ray structures of other CAOs: *Escherichia coli* amine oxidase (ECAO) [128-132], pea seedling amine oxidase (PSAO) [133], *Hansenula polymorpha* amine oxidase (HPAO) [134, 135] and *Arthrobacter globiformis* amine oxidase (AGAO; Figure 6) [112, 136, 137] even though the sequence homology is in the range of only 15 - 24% (Figure 4).

The structures of copper amine oxidases [112, 113, 128-137] form the shape of a mushroom cap composed of three domains (Figure 5 and 6). Subunit domains in BPAO have been proposed from differential scanning calorimetry (DSC) experiments [110, 138] which suggest that BPAO has 3 domains per monomer. In the case of ECAO, the presence of an additional domain (D1) at the N terminal region (85 residues) that forms the stalk of the mushroom is observed [128-132]. The BPAO crystal structure does not show a D1 domain (Figure 5) [113].

All structures show a large D4 domain at the C terminal region (440 residues) that forms the bulk of the mushroom cap. It is composed of two antiparallel β -sheet sandwiches with 10 and 8 β -strands each (Figure 5). This forms the bulk of the subunit interface and contains the active site residues (Figure 6). Domains D2 and D3 are homologous in sequence (100 residues each) and similar in structure [113, 133, 134, 136]. They are located at the outer edge of the mushroom cap (Figure 6a).

In each D4 domain, there are two pairs of β -hairpin arms that extend across to the other monomer (Figures 6c and 6d). One pair (Arm I) links the two active sites and lies on top of the D4 cap toward the peripheral D3 domain while the other (Arm II) lies at the

bottom of the mushroom cap in a groove and likely provides stability to the dimer [136]. A comparison of arms I and II (Figure 7) shows that the length and orientation of arm I is more conserved while arm II is more variable [136].

Sequence homology of copper amine oxidases by ClustalW 1.81 [126] reveals a conserved disulfide bond for BPAO between C403 and C429 (Figure 5) in each monomer. In addition, there is a second disulfide bond between cysteines 197 and 198 in each monomer [113]. Analysis of the surface cysteines in BPAO (PBD: 1TU5; [113]) by Rasmol 2.7.2.1 [139] shows the presence of only one free cysteine residue (C94) per monomer, in contrast to earlier studies [140]. Suva [141] and Zeidan [142] further found that the addition of substrate liberates an essential sulfhydryl residue which is located in the restrictive active site pocket with no direct interaction to the copper, suggesting that they are at least 14 Å apart [142].

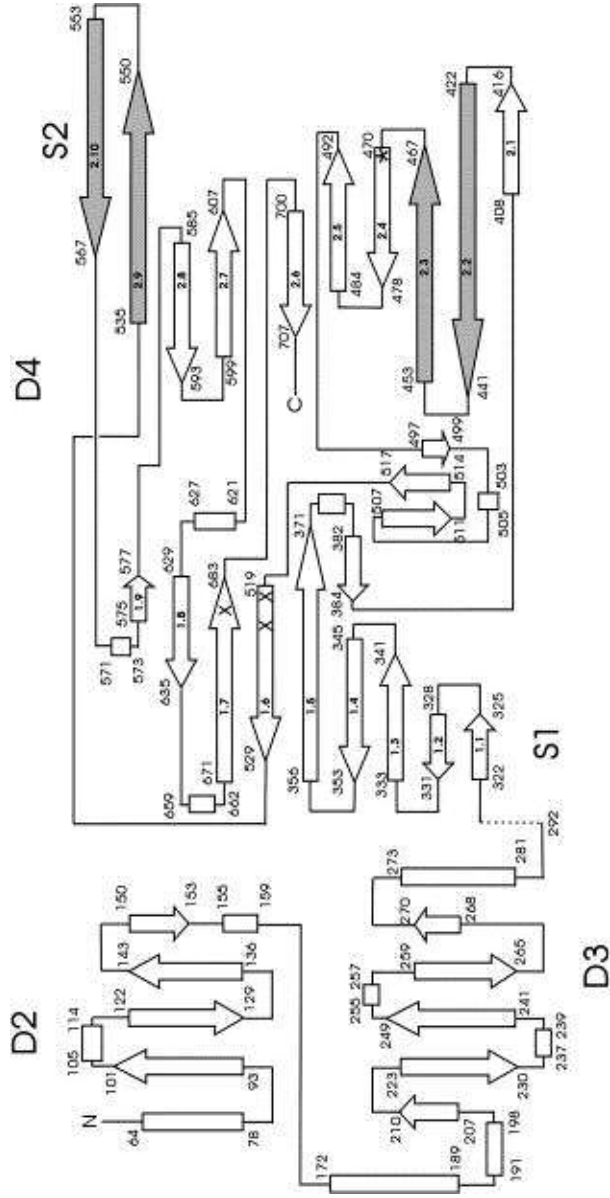


Figure 5: Overall topology of the BPAO monomer. Domains are numbered from D2 to D4, in agreement with the ECAO structure. Numbers indicate the beginning and end of secondary structure elements. An asterisk (*) shows the position of TPQ, and X that of the His residues coordinating Cu(II). Grey arrows highlight the position of the two long arms that protrude from domain D4 of one monomer to wrap around the other. Since the two β -sheets are heavily bent, the two arms protrude in the same direction, despite appearing anti-parallel in the topology. Taken from Lunelli M, Di Paolo ML, Biadene M, Calderone V, Battistutta R, Scarpa M, Rigo A, Zanotti G (2005) Crystal structure of amine oxidase from bovine serum. *J Mol Biol.* 346(4):991-1004 [113]

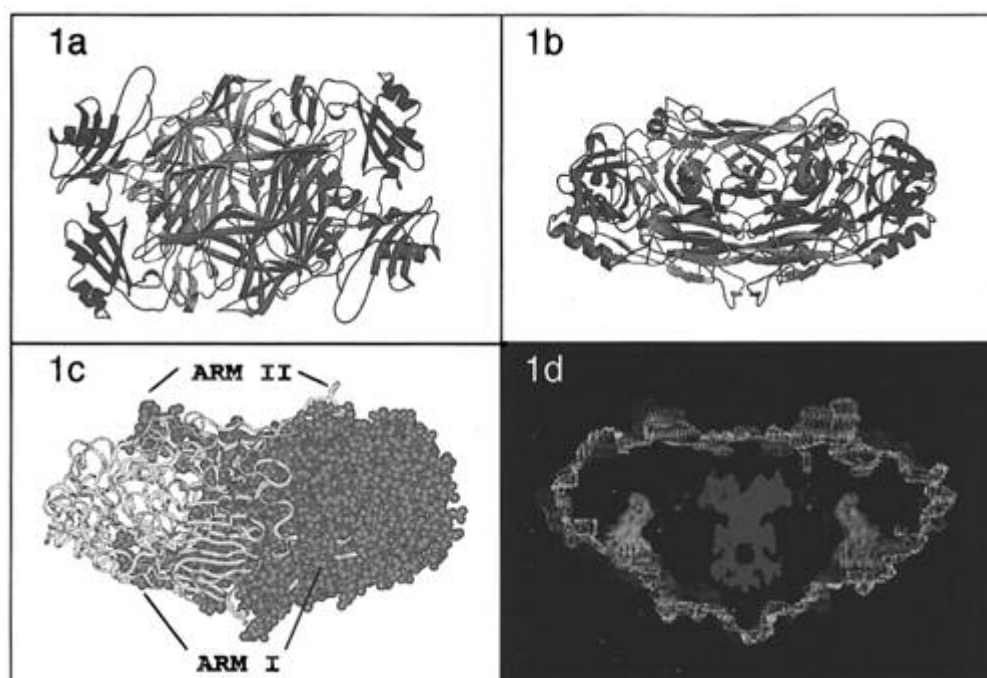


Figure 6 Four views of the *A. globiformis* amine oxidase (AGAO) molecule. (a) Ribbon diagram of the dimer viewed along its 2-fold symmetry axis: domain 2; domain 3; and domain 4. The Cu atom at the active site of each subunit is shown as a sphere. (b) The AGAO dimer viewed along a direction perpendicular to the 2-fold symmetry axis. (c) The AGAO dimer in an orientation similar to that in panel b. One subunit is represented as a ribbon and the other as a space-filling model. The two arms that originate from each subunit and grasp the other subunit are seen clearly. Much of arm II (the upper arm in this orientation) is exposed, whereas arm I fits into a groove on the surface of the other subunit and is only partly visible. (d) A section through the AGAO dimer, showing the molecular boundary, the solvent-filled cavity, and the channels to the two active sites. The Cu atom, the side chains of the three Cu-binding histidine residues and the TPQ cofactor at each of the two active sites are also shown. The TPQ lies on the side of the active site remote from the solvent-filled cavity. The molecular boundary is a 1.4 Å rolling-sphere surface.

Taken from Wilce MCJ, Dooley DM, Freeman HC, Guss JM, Matsunami H, McIntire WS, Ruggiero CE, Tanizawa K and Yamaguchi H. (1997) Crystal structures of the copper-containing amine oxidase from *Arthrobacter globiformis* in the holo and apo forms: implications for the biogenesis of topaquinone. *Biochemistry* 36(51): 16116-33 [136]

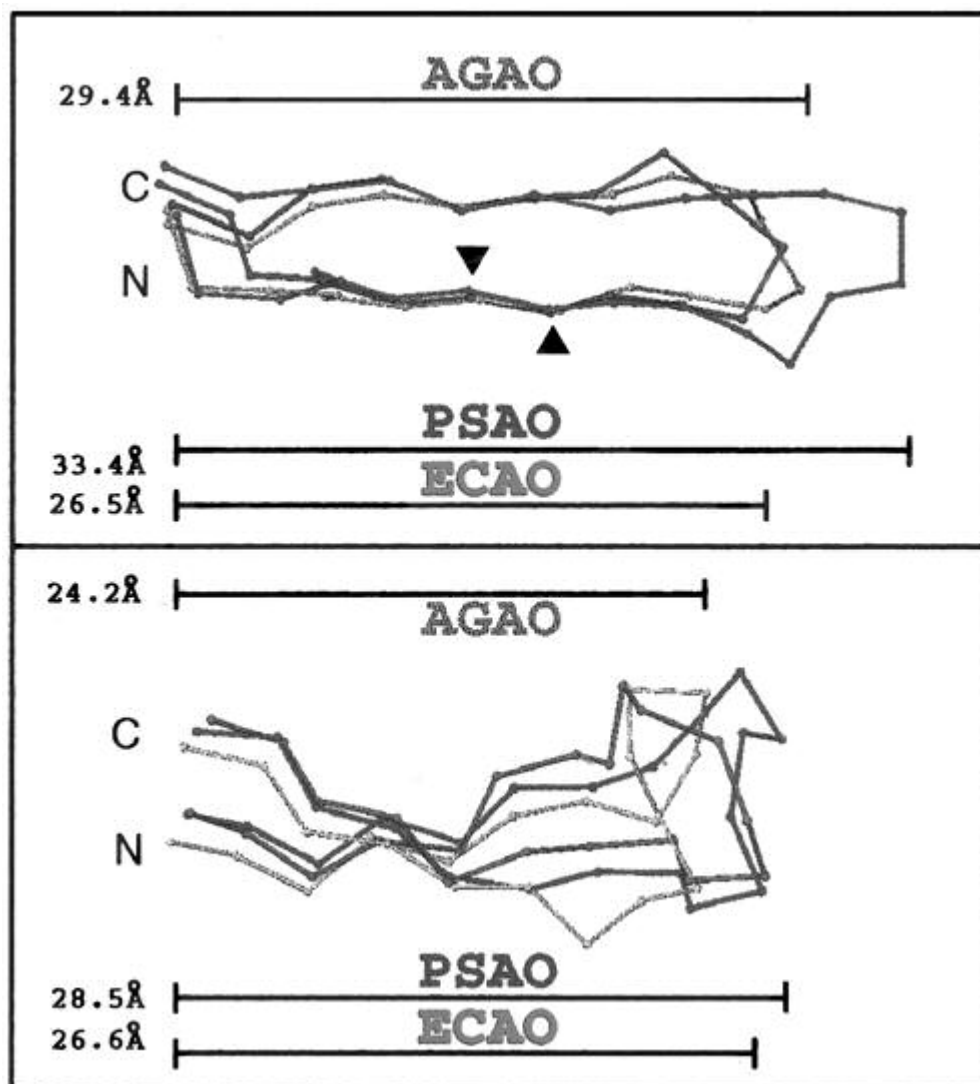


Figure 7 Semiquantitative comparison between the intersubunit arms in PEA0, PSAO, and ECAO. The orientation is approximately the same as that in Figure 6c (upper, arm I; lower, arm II). The alignment was obtained by superimposing the three entire molecules. The central portions of arm I are almost exactly aligned so that the residues corresponding to Lys354 and His355 in AGAO occupy the same position in all three proteins. The conformations of arm II are more varied. The lengths of the two arms (in Å) are measured from corresponding residues at the left-hand edge of the Figure.

Taken from Wilce MCJ, Dooley DM, Freeman HC, Guss JM, Matsunami H, McIntire WS, Ruggiero CE, Tanizawa K and Yamaguchi H. (1997) Crystal structures of the copper-containing amine oxidase from *Arthrobacter globiformis* in the holo and apo forms: implications for the biogenesis of topaquinone. *Biochemistry* 36(51): 16116-33 [136]

1.4.1.4.1 Active Site

The entrance to the active site is located at the junction of the D3 and D4 domains (Figure 6d). A 12 -18 Å channel leads from the molecular surface to the active site cofactors, copper and TPQ (Figures 6d and 8), which may facilitate the movement of reactants and products to and from the active site [136]. It is composed of residues from the D2, D3 and D4 domains as well as residues of the hairpin arm I belonging to the other subunit (Figure 9A). The channel facilitates the recognition of substrates by the negative charges in the channel (Figure 9B) and the presence of a gating mechanism (Y383 in BPAO [113], Y296 in AGAO [136], Y381 in ECAO [129] and F298 in PSAO [133]) that allows selective access into the active site (Figure 10). At the end of the channel is the active site base (D385 in BPAO [113], D319 in HPAO (Figure 8, [135]) and D383 in ECAO [129]) that abstracts a proton from the amine substrate during the reductive half reaction (Equation 15).

The TPQ cofactor in the active site is quite flexible. It can rotate along the C α -C β bond to form either the active (Figure 11A) or inactive (Figure 11B) conformation. In the active protein (Figure 11A), the copper atom or copper (II) ion is ligated to the three invariant histidine residues and two water molecules while in an inactive protein, TPQ is a ligand to the copper (II) ion along with the three invariant histidine residues (Figure 11B). The active site of apo-AGAO (Figure 11C) shows the precursor tyrosine in an orientation similar to that of active TPQ. Even though copper amine oxidases oxidize a variety of primary amine substrates, the active site conformations are quite similar.

In addition to the intersubunit interaction of the arms (Figure 6C) and its presence in the active site channel (Figure 9A), also noteworthy is that the active site cofactor TPQ

(Y470) directly follow the β -ribbon arms in amino acid sequence (422-467) (Figure 5). Therefore, subtle changes in the conformation of one active site can be transferred to the active site on the other subunit.

Intersubunit interactions are decreased due to the presence of a large solvent filled cavity between the D4 domains of the two subunits (Figure 6d). It can hold from 80 (ECAO) to 270 (PSAO) solvent molecules [136]. In BPAO, three small channels connect the internal cavity to the external solvent and they have been proposed to provide a pathway for the O_2 substrate and H_2O_2 product [113].

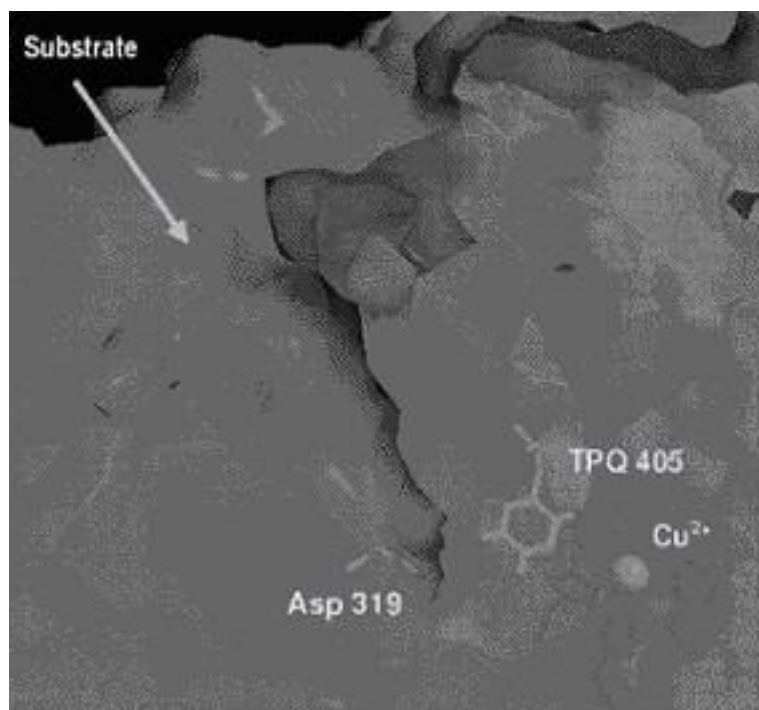


Figure 8: Substrate entry channel for HPAO. The enzyme shows the TPQ cofactor in its active orientation, below the translucent molecular surface. Also shown are the active site base (D319) and the copper (II) ion. This diagram was made using GRASP and rendered with RASTER3D.

Taken from Chen Z, Schwartz B, Williams NK, Li R, Klinman JP, Mathews FS (2000) Crystal structure at 2.5 Å resolution of zinc-substituted copper amine oxidase of *Hansenula polymorpha* expressed in *Escherichia coli*. *Biochemistry* 39(32): 9709-17 [135]

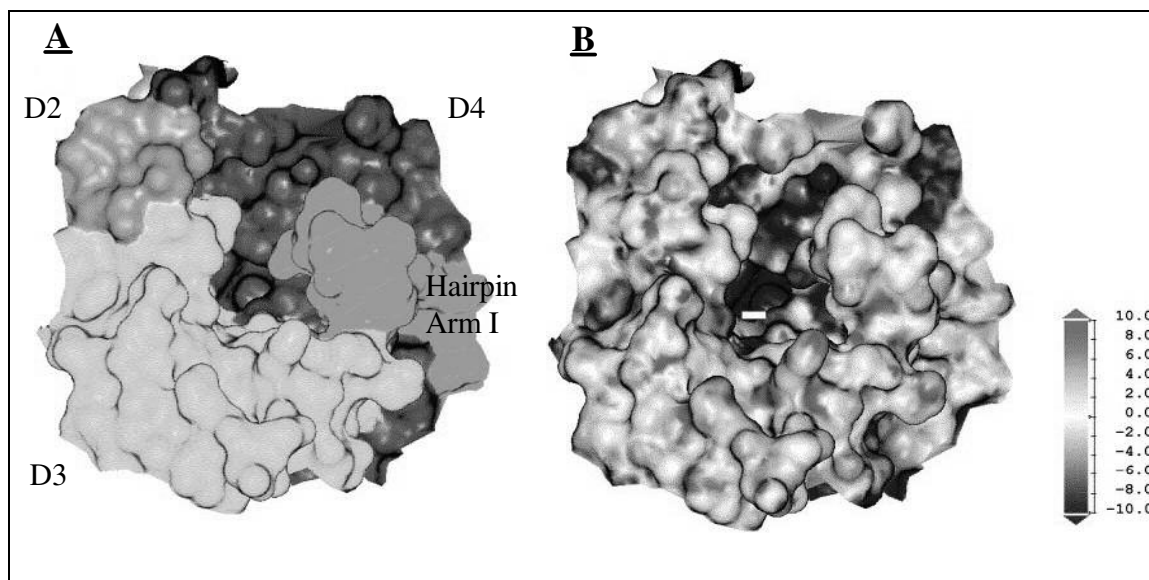


Figure 9: Surface of the cavity leading to the active-site in BPAO. (A) The residues are labeled according to the domains (D2, D3 and D4) or residues of the hairpin belonging to the other subunit (Hairpin Arm I). (B). The surface is grayscale according to the electrostatic potential; units are kT/e (corresponding to $0.593 \text{ kcal}/(\text{mole}\cdot e)$ at 25°C). Negative and positive charges are randomly distributed on the surface of the channel and there is an electronegative gradient within the channel cavity.

Taken from Lunelli M, Di Paolo ML, Biadene M, Calderone V, Battistutta R, Scarpa M, Rigo A, Zanotti G (2005) Crystal structure of amine oxidase from bovine serum. *J Mol Biol.* 346(4): 991-1004 [113]

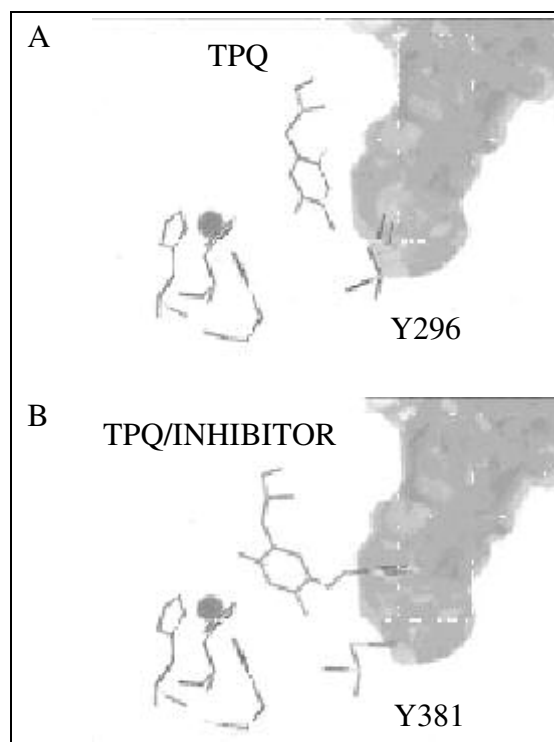


Figure 10: Channel from the molecular surface to the active site. (A) The channel in relation to the active site of holo-AGAO. At the end of the channel, the Tyr296 gate is shown in the closed position. The copper (II) ion and its histidine ligands are also shown. (B) A composite diagram incorporating the channel and Cu site of holo-AGAO from panel A and the TPQ-2-hydrazinopyridine inhibitor complex of ECAO. The Tyr381 gate in the inhibitor complex is shown in the open position.

Taken from Wilce MCJ, Dooley DM, Freeman HC, Guss JM, Matsunami H, McIntire WS, Ruggiero CE, Tanizawa K and Yamaguchi H. (1997) Crystal structures of the copper-containing amine oxidase from *Arthrobacter globiformis* in the holo and apo forms: implications for the biogenesis of topaquinone. *Biochemistry* 36(51): 16116-33 [136].

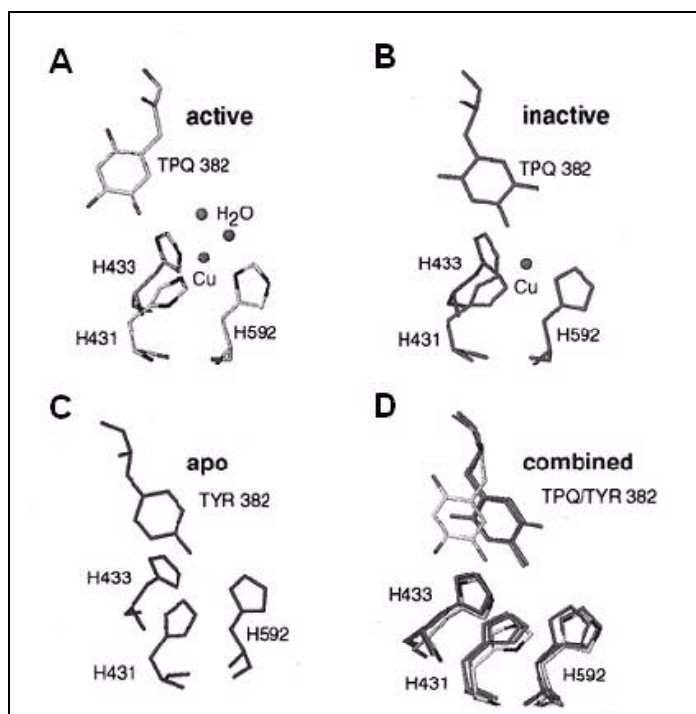


Figure 11 Active site conformation. (A) The active form of holo-AGAO, (B) the inactive form of holo-AGAO, (C) apo-AGAO, and (D) a superposition of the three forms of AGAO. The Cu atom and the two coordinated H₂O molecules are shown as spheres. Taken from Wilce MCJ, Dooley DM, Freeman HC, Guss JM, Matsunami H, McIntire WS, Ruggiero CE, Tanizawa K and Yamaguchi H. (1997) Crystal structures of the copper-containing amine oxidase from *Arthrobacter globiformis* in the holo and apo forms: implications for the biogenesis of topaquinone. *Biochemistry* 36(51): 16116-33 [136].

1.4.1.5 Substrates

BPAO has a high affinity for substrates that vary in size and bulk [143]. Table 4 shows the relative rates of substrate oxidation, with the highest rate normalized at 100 for spermine and spermidine (Figure 15). Benzylamine is used for activity assays and Table 11 shows the K_m for BPAO which is about 1.5-2mM with this substrate.

Further probing of the active site has elucidated the interactions between BPAO and the substrates. Di Paolo *et al* [144] found that there are three important amino acid residues in the active site of BPAO that enable the physiological substrates, spermine and spermidine, to dock (Figure 16). They are Asp 385 (G), and His 442 (I) and Asp 445 (L) which belong to the hairpin arm I of the other subunit [113]. In general, it was found that as the chain length of the substrate increases, so does its affinity for BPAO and the presence of a positively charged N group at position 5, decreases the affinity whilst the N at position 10 increases the affinity [113, 144]. Hence, substrates bind at different regions of the active site based on their structure and charge distribution.

Table 4
Relative Rates of Oxidation of Various Substrates by BPAO

Substrate	Relative rate (%)
Spermine	100
Spermidine	100
Benzylamine	40
Butylamine	40
Kynuramine	20
Phenylethylamine	20
Tyramine	15

Adapted from Mondovi B, Turini P, Befani O, Sabatini S (1983) Purification of bovine plasma amine oxidase. *Methods Enzymol* 94: 314-8 [143].

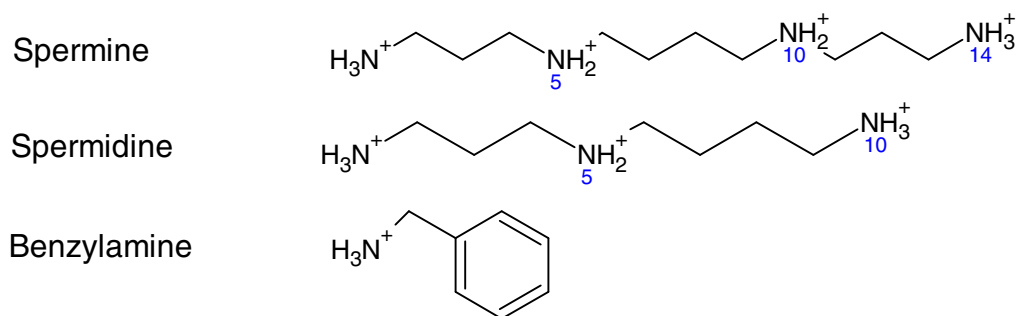


Figure 12: Structures of BPAO substrates.

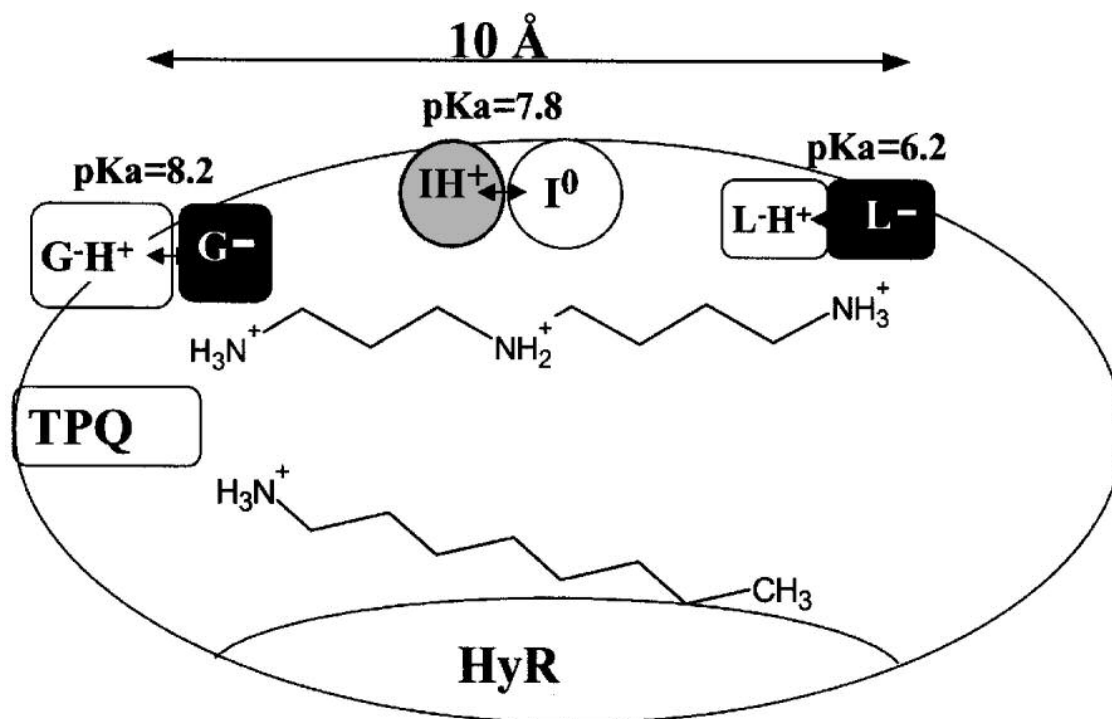


Figure 13: Active-site model of BSAO. This active-site model has been proposed on the basis of experimental data. $G-H^+$ (Asp 385), IH^+ (His 442') and $L-H^+$ (Asp 445') are residues that interact with the substrate according to its structure [113]. The apostrophe indicates that the residue belongs to the arm of the other subunit [113]. The pK_a and charge of these groups and their approximate location are reported on the basis of experimental results. HyR is a hydrophobic region where highly hydrophobic substrates may bind. In this model, the two different binding modes are shown. Apolar substrates, such as nonylamine, interact with the hydrophobic binding region. On the other hand, polyamines (such as spermidine) interact, by electrostatic interactions, with the charged region of the active site where the three protonable residues (deprotonated in the enzyme active form), that control the docking of these polyamines, are localized. Taken from Di Paolo ML, Stevanato R, Corrazza A, Vianello F, Lunelli L, Scarpa M and Rigo A (2003) Electrostatic compared with hydrophobic interactions between bovine serum amine oxidase and its substrates. *Biochem J* 371: 549-556 [144].

1.4.1.6 Inhibitors

Noncompetitive inhibitors of BPAO include the biologically important amines tryptamine, serotonin and histamine [143]. Table 5 shows the competitive inhibitors of BPAO, which include secondary or tertiary amines, amines with constituents at the α carbon position, substrate analogs, some alkaloids and copper chelating agents [97]. Carbonyl reagents such as phenylhydrazine irreversibly inhibit BPAO.

Inhibitors can be classified into two categories according to a mode of action:

- 1) inhibits copper;
- 2) inhibits TPQ either reversibly or irreversibly.

Table 6 summarizes some inhibitors of BPAO which effects either the copper or TPQ. Most notably, low benzylamine concentrations (4mM), inactivates BPAO by modification of the TPQ cofactor to a benzoxazole derivative in a time dependant manner (19hr) [145].

Table 5
Relative rates of competitive inhibitors on BPAO activity.^a

Added Amine	Relative Activity (%)
None	100
Piperidine	59
2,2,6,6-Tetramethylpiperidine	91
4-Hydroxy-2,2,6,6-tetramethylpiperidine	56
Aniline	58
2-Amino-2-methyl-1-propanol	90
2(4-Aminophenyl)ethanol	90
2,2,6,6-Tetramethyl-4-piperidone	68
4-Aminobenzoylhexylamide	67
4-(4-Aminophenyl)butyric acid	52

^a Activity measurements were carried out in 40 mM Hepes, pH 7.0 at 37°C with 0.1M spermine as the substrate. The inhibitor concentration was 0.01M.

Adapted from Vianello F, Di Paolo M. L., Zennaro L, Stevanato R and Rigo A. (1992) Isolation of Amine Oxidase from Bovine Plasma by a two-step procedure. *Protein Expression and Purification* 3: 362-367 [146].

Table 6
Inhibitors of BPAO

Inhibits copper	Reversibly inhibits TPQ	Irreversibly inhibits TPQ
Azide [147]	Aniline [146]	2-Butynamine [154]
Sulfide [147]	4-Amino TEMPO [148]	2-Cl-Allylamine, Allylamine [155, 156]
	Dopamine [149]	3-Pyrrolines [157]
	Hydrazine and derivatives [150]	Propargylamine [154, 155]
	Ions: Ca, K, Na and Cs [151]	Arylhydrazines [150]
	Pyrazole derivatives [152]	Benzylamine [145, 158]
	Tranylcypromine (TCP) [153]	

1.4.1.7 Conformational Stability of BPAO

Previous studies have focused on defining the active site structure of BPAO to elucidate the role of the copper cofactor [106-111] but few studies thus far have focused on the conformational stability of the protein. Early conformational studies monitored the association of the subunits [159, 160]. Turini *et al* [160] showed by ultracentrifugation that the addition of GuHCl dissociates the homodimer and further addition of 0.1% β -mercaptoethanol unfolds the monomers. Ishizaki *et al* [161] monitored the thermal denaturation of the secondary structure changes from the far-UV circular dichroism.

Since BPAO is dimeric and has multiple domains [110, 113, 138], it is an appropriate protein model to study the unfolding and dissociation pathways. This information will contribute to our understanding of the folding mechanisms of high molecular weight oligomeric proteins.

1.5 Methods to study protein unfolding and dissociation

Numerous biophysical techniques, including fluorescence, gel electrophoresis, liquid chromatography and residual activity, are available to study protein folding [162]. In order to investigate the unfolding pathway of BPAO from the native to denatured conformation, a variety of techniques were used. The timescale over which the experimental technique averages, can provide information about both local and global protein fluctuations. Thus, employing several experimental approaches allows characterization of the complex unfolding pathways. Here, global changes were followed spectrophotometrically and by HPLC and PAGE. Local perturbations are revealed by spectroscopic methods and specific activity measurements.

1.5.1 Fluorescence

Fluorescence is the emission of absorbed light which occurs at a lower energy (longer wavelength) than excitation (Figure 14). Upon excitation by light, the electron in the fluorophore is promoted from the ground state into the excited state in about 10^{-15} sec [163]. In the ground state, the electrons tend to occupy the lowest vibrational level (S_0). Depending on the wavelength and therefore energy of excitation, the electron is promoted to higher vibrational levels of the (generally) first (S_1) excited state. The electron in the fluorophore relaxes to the lowest vibrational energy level of S_1 *via* internal conversion which occurs within about 10^{-12} sec. Fluorescence is the emission of light resulting from the transition from the lowest vibrational energy level of S_1 back to S_0 . As a consequence, the emission frequencies are independent of the excitation wavelength. Emission occurs on a timescale of about 10^{-8} sec (or about 10ns). The absorption spectrum shows the

absorption of energy by the molecule as a function of wavelength and reveals the vibrational levels of the excited state. The emission spectra show vibrational levels of the ground state as a function of wavelength (λ). For many molecules, the absorption spectrum is a mirror image of the fluorescence emission spectrum.

Fluorescence spectroscopy can monitor protein conformational changes [164]. It can report on tertiary conformational changes of proteins from the sensitivity of measured parameters from steady state (e.g. integrated intensities (I)) or time resolved measurements (e.g. fluorescence lifetime (τ) and rotational correlation times (ϕ)) arising from either intrinsic (e.g. tryptophan, tyrosine, cofactors) or extrinsic (e.g. pyrene maleimide, ANS, bis-ANS) fluorophores which are sensitive to their immediate local protein and/or solvent environments.

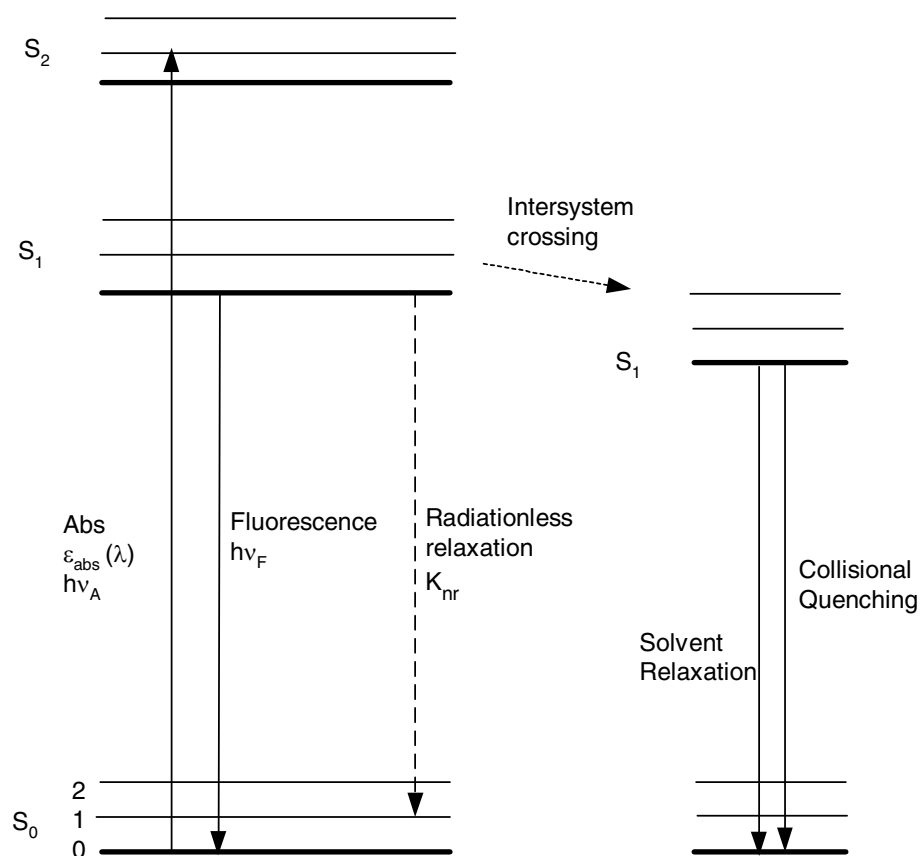


Figure 14: Jablonski diagram. S_0 , S_1 and S_2 are the different electronic levels and 0, 1 and 2 are the different vibrational energy levels within each electronic level. Shown are absorbance excitations, and emissions *via* fluorescence, radiationless relaxation, solvent relaxation and collisional quenching.

1.5.1.1 Fluorophores

The many kinds of fluorophores are categorized according to whether they are naturally occurring (intrinsic), or chemicals added or conjugated to a non-fluorescing sample (extrinsic).

1.5.1.1.1 Intrinsic Fluorophores

Naturally occurring fluorophores include the aromatic amino acids, tryptophan, tyrosine and phenylalanine (Figure 15A), and some cofactors including TPQ, reduced nicotinamide adenine dinucleotide (NADH), oxidized flavins (FAD, flavin adenine dinucleotide, FMN, flavin mononucleotide), pyridoxal phosphate and chlorophyll [163]. Of the fluorescent amino acids, only tryptophan can be selectively excited at 295nm.

There are two vibrational emission bands in tryptophan, ^1L_a and ^1L_b [163]. ^1L_a is sensitive to solvent so tryptophan fluorescence is highly sensitive to its local environment [163, 165]. In folded proteins, tryptophan emission occurs between 335-345nm [71, 75, 79, 82, 166] with a quantum yield of 0.26 [166]. However, in an unfolded conformation, the emission is at 355nm [71, 75, 79, 82, 163, 166] and the quantum yield decreases to 0.08 [166]. This red-shift in solution is due to hydrogen bonding of the N-H of the indole ring to solvent [167]. The tryptophan fluorescence may also be quenched by introduced molecules such as iodide, acrylamide and oxygen (Section 1.5.1.2.1).

Different rotomers of tryptophan exist in the ground state, leading to multiple lifetimes for a single tryptophan residue [168]. In buffer, tryptophan has a long and short lifetime of about 3ns and 1ns respectively [169]. In proteins, the long tryptophan lifetime increases to 5-6ns [166, 170, 171]. The rotations of tryptophan residues in proteins

exhibit both a short (0.1-0.4 ns) and long (>9 ns) rotational components, representing local rotations of a short polypeptide segment (or domain) and the whole macromolecule, respectively [166, 172]. The limiting anisotropy value for tryptophan in proteins is reported to be 0.2 [166 170, 171].

Spectral information from multiple tryptophan residues represent an average signal from all the residues and are often not easily resolved [168, 173].

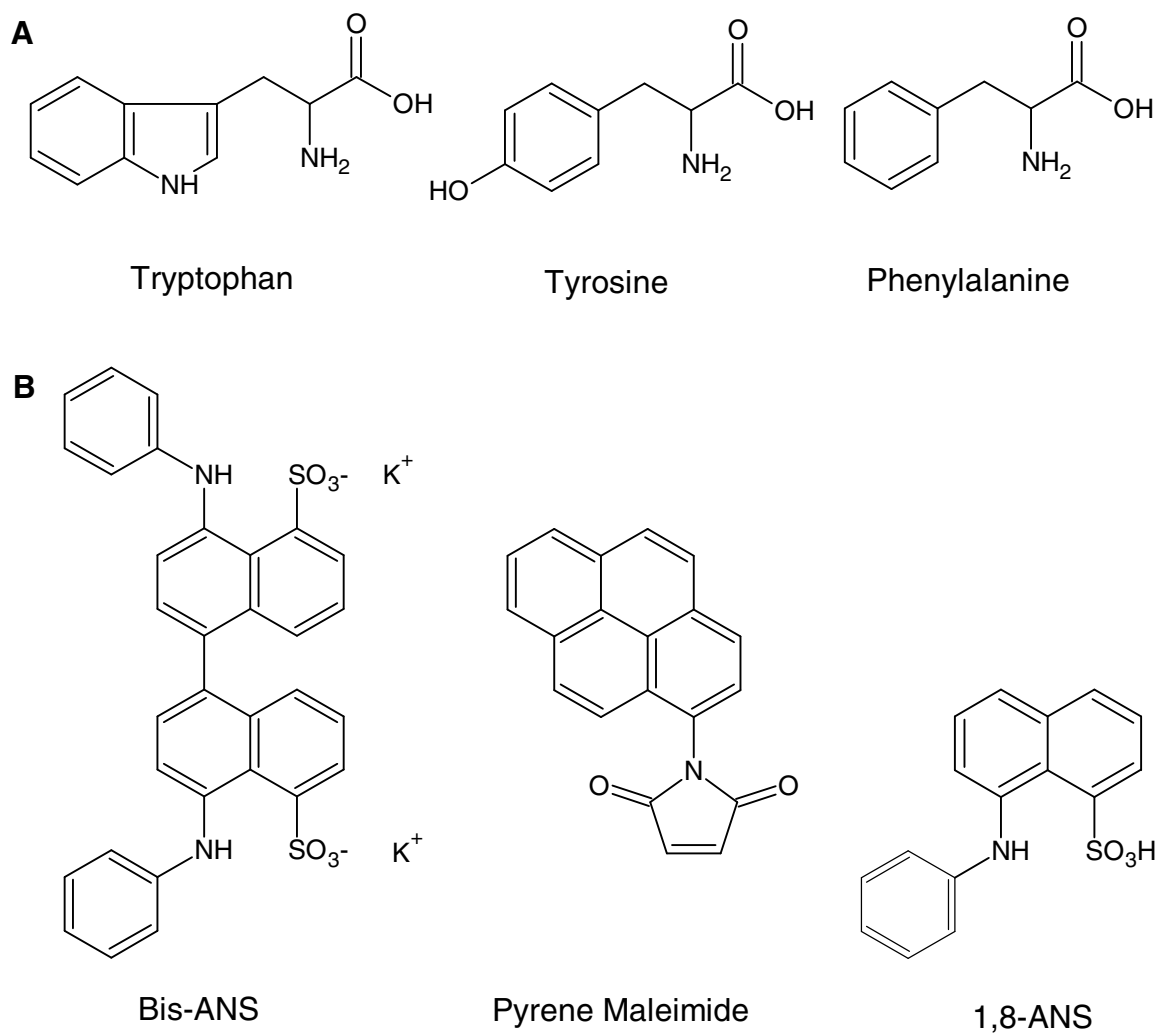


Figure 15: Structures of intrinsic (A) and extrinsic (B) fluorophores used in protein studies. The extrinsic fluorophores used in the studies described here are bis-ANS, pyrene maleimide and 1,8-ANS.

1.5.1.1.2 Extrinsic fluorophores used in this study

1.5.1.1.2.1 Covalently bound probes

The sulfhydryl and amino groups are sites available for covalent attachment of a fluorophore to a protein. One such probe is N-(1-pyrene) maleimide [174] (hereafter referred to as simply pyrene maleimide (PM); Figure 15B), which covalently binds to the thiol group of cysteine residues. This probe offers some unique spectroscopic parameters for studies of altered protein conformation, including:

1) Pyrene maleimide binds to cysteine residues to form a covalent bond [175, 176] and therefore has a well defined location on the macromolecule.

2) Pyrene maleimide is not fluorescent until bound to a thiol group [174, 175].

3) Pyrene has well defined vibronic bands that are sensitive to solvent at 374, 384 and 394nm [177-179]. Pyrene vibrational emission peak III and peak I correspond to peaks at 384 nm and 374 nm in protein labeled with pyrene maleimide. The intensity ratio of vibrational peaks (I_3/I_1) can provide information about the polarity of the probe environment [177-180]. A ratio value of 0.5 – 0.8 reflects a polar microenvironment, while 1.65 – 1.75 reflects a more nonpolar hydrocarbon microenvironment for the pyrene label [178].

4) Monomeric pyrene maleimide exhibits a long fluorescence lifetime (>30 ns) [181-184], and is therefore an attractive probe for studying the rotational motions of large molecular weight proteins. Changes in rotational volumes as a result of protein conformational changes can be monitored through measurement of the fluorescence emission anisotropy (Section 1.5.1.3). The limiting anisotropy, r_0 , for PM is reported to be ~0.2 [182].

5) Pyrene maleimide can form excited state dimers (excimers) from specific interactions between an excited monomer and a ground state monomer [185] when the monomers are located within 10Å [186] and the pyrene ring systems are stacked 3.5Å apart [187]. The fluorescence emission spectrum for pyrene excimers (from 450 to 470 nm) is a broad, featureless and red shifted from the highly structured monomeric vibrational energy level emissions (370-400 nm). Formation of excimers can thus provide information about subunit or domain interactions [181, 188, 189].

6) Pyrene maleimide can serve as an acceptor or donor for Förster resonance energy transfer (FRET) studies. The excitation spectrum for pyrene maleimide monomer exhibits an absorbance maximum at 340nm, which overlaps favorably with the intrinsic tryptophan emission maximum at 335-345nm. The Förster distance (R_0), for 50% efficiency of energy transfer between pyrene maleimide and tryptophan, is on the order of 20-30Å [190, 191]. The efficiency of transfer (E) from tryptophan to pyrene maleimide can vary significantly with distance, giving information about altered protein conformations. Additionally, pyrene maleimide can serve as an attractive FRET donor for other fluorescent probes [192].

1.5.1.1.2.2 Non-covalently bound fluorophores

Rather than covalent binding, other probes can bind through non-covalent or hydrophobic interactions to a macromolecule [193-195]. Examples are 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS or ANS) a dimer of ANS, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS) (Figure 15B). These probes possess favorable characteristics for fluorescence studies, including:

1) The fluorescence emission intensity is very sensitive to the polarity of the microenvironment. The aromatic groups of fluorophores preferentially associate with hydrophobic sites on a macromolecule and result in an increase in the fluorescence emission intensity. For example, the quantum yield, Φ , of 1,8-ANS in water is 0.004 [196, 197], but when bound to a hydrophobic site on a protein, increases appreciably to 0.98 [198].

2) They provide an excellent approach for estimating the number of probes bound to hydrophobic sites on proteins. Depending on the concentrations of free and bound probe, and protein, analysis of the fluorescence emission intensities on binding (Section 1.5.3.2, Equations 45 and 47) enables the calculation of the number of probes bound (n) and the corresponding dissociation constants (K_D) for ANS and bis-ANS (Table 7) which provides a measure of the hydrophobicity of the protein [21].

3) Additionally, these hydrophobic probes have been used extensively for determining the presence of protein folding intermediates, specifically the molten globule. As discussed, the molten globule has native-like secondary structure, but a fluctuating tertiary structure that has more exposed hydrophobic patches (Section 1.2.1) [13-18]. The enhanced binding of anilino-probes can thus provide evidence for this conformational intermediate [16, 20-23].

1.5.1.2 Fluorescence Intensity Measurements

During protein unfolding, the dielectric of the immediate environment surrounding a fluorophore can change. Such effects can give rise to shifts in the fluorescence emission spectra and/or altered fluorescence intensities. Very often, measurement of small changes in the fluorescence intensity at one particular emission wavelength is difficult. An alternate approach is to determine the center of spectral mass (CSM) of the emission spectrum. The CSM represents the weighted average of the entire fluorescence emission spectrum. It is calculated by the equation:

$$CSM = \frac{\sum_i \lambda_i F_i}{\sum_i F_i} \quad (17)$$

where λ is the wavelength of a particular fluorescence intensity (F) [90, 199]. This method of analysis is favored over a single wavelength intensity measurement because it eliminates small fluctuations in the intensity by analyzing over a wide spectral range.

1.5.1.2.1 Fluorescence Emission Quenching

Quenchers decrease the fluorescence emission intensity of a fluorophore [163, 200]. They include a variety of molecules that interact with the fluorophore in the ground (static quenching) or the excited states (dynamic quenching, e.g. O₂, acrylamide, iodide). In proteins, electrostatic and hydrophobic interactions of the fluorophore with its local protein or solvent environment can cause quenching, which is relieved upon denaturation.

The quenching of the fluorescence intensity may be described by the linear Stern-Völmer equation [163, 200]:

$$\frac{F_0}{F} = 1 + K_{SV} [Q] \quad (18)$$

where F and F_0 are the fluorescence intensities in the presence and absence of quencher Q , respectively, and K_{SV} is the Stern-Völmer quenching constant.

Static quenching arises from ground state interactions between the fluorophore and quencher to form a complex that is non-fluorescent [163]. In such cases, the Stern-Völmer quenching constant, K_{SV} , is equivalent to the association constant, K_A , for complex formation between the fluorophore and quencher.

Dynamic (or collisional) quenching results from excited state interactions between the quencher and fluorophore to form an excited state complex that returns to the ground state without emitting a photon [163]. Here the change in fluorescence intensity is equivalent to the change in lifetime in the presence (τ) and absence (τ_0) of quencher according to the equation [163]:

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} \quad (19)$$

and the Stern-Völmer Equation (18) is rewritten as Equation 20, where K_{SV} is equal to $k_q\tau_0$ and k_q is the bimolecular quenching constant:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + k_q \tau_0 [Q] \quad (20)$$

In cases where the plot of Stern-Völmer equations are non-linear and reaches a limiting fluorescence intensity with addition of quencher, data may be analyzed using a modified Stern-Völmer (Lehrer) equation:

$$\frac{F_0}{F_0 - F} = \frac{1}{[Q]f_a K_Q} + \frac{1}{f_a} \quad (21)$$

where K_Q is the quenching constant for the exposed fluorophores (such as tryptophan) on the surface of a protein and f_a is the fraction of the initial fluorescence that is accessible to the quencher [163, 201, 202].

1.5.1.3 Fluorescence Emission Anisotropy (EA)

The rotational motions of a fluorophore may be expressed by the fluorescence emission anisotropy, $\langle r \rangle$. The emission anisotropy of a fluorophore [163, 203, 204], following excitation using polarized light, is calculated by:

$$\langle r \rangle = \frac{I_{VV} - GI_{VH}}{I_{VV} + G 2I_{VH}} \quad (22)$$

where the subscripts V (vertical) and H (horizontal) refer to the direction of the excitation (first) and emission (second) polarizers (Figure 16) and G is the grating factor [205]

corrects for differences in the instrument emission detection sensitivity for vertical and horizontal excitation light (Equation 23).

$$G = \frac{I_{VH}}{I_{HH}} \quad (23)$$

The total fluorescence emission intensity(s) is equal to the sum of polarized interactions (the denominator of Equation 22),

$$\text{Total Intensity} = I_{VV} + G.2I_{VH} \quad (24)$$

For a fluorophore with colinear absorption and emission dipoles, and in a rigid environment, the maximum anisotropy value that can be obtained (r_0) is 0.4, and r_0 is -0.2 when the absorption and emission dipoles are at right angles to each other [163]. The value of the limiting anisotropy (r_0) thus reflects the angle between the absorption and emission dipoles. For tryptophan, r_0 is about 0.2 (λ_{ex} = 295nm) [171]. Measured values for r_0 in a rigid matrix are often less than the expected theoretical value because of fast intrinsic depolarizing motions of the fluorophore [163].

Measurement of the emission anisotropy is a valuable approach for discriminating between dimer unfolding and dissociation properties of a multisubunit protein, since the rotational properties of a monomer and those of the unfolded dimer will be very different [206]. However, the value of the anisotropy is sensitive to the fluorescence lifetime of the fluorophore (τ). The lifetime must be sufficiently long to be able to experimentally

observe slow rotational properties (measured by the rotational correlation time, ϕ) of a large molecular weight protein.

For a spherical protein, the rotational motions (ϕ) can be estimated from the measured emission anisotropy values ($\langle r \rangle$) through the Perrin equation:

$$\frac{r_0}{\langle r \rangle} = 1 + \frac{\tau}{\phi} \quad (25)$$

where $\langle r \rangle$ and r_0 are the measured emission anisotropy and limiting anisotropy values, respectively. The rotational correlation time, ϕ , defined by the Stokes-Einstein equation, is related to the molecular weight by:

$$\phi = \frac{\eta V}{RT} = \frac{\eta M(\nu + h)}{RT} \quad (26)$$

where η is the viscosity of the solution (0.89×10^{-2} P in water at 25°C), R is the gas constant (8.314×10^7 erg.K⁻¹.mol⁻¹), T is the temperature (in Kelvin) and the protein volume, V , is equivalent to the molecular weight (M) multiplied by the sum of the specific volume (ν , near 0.73 ml/g) and hydration (h , typically 0.2g H₂O per g protein) [163].

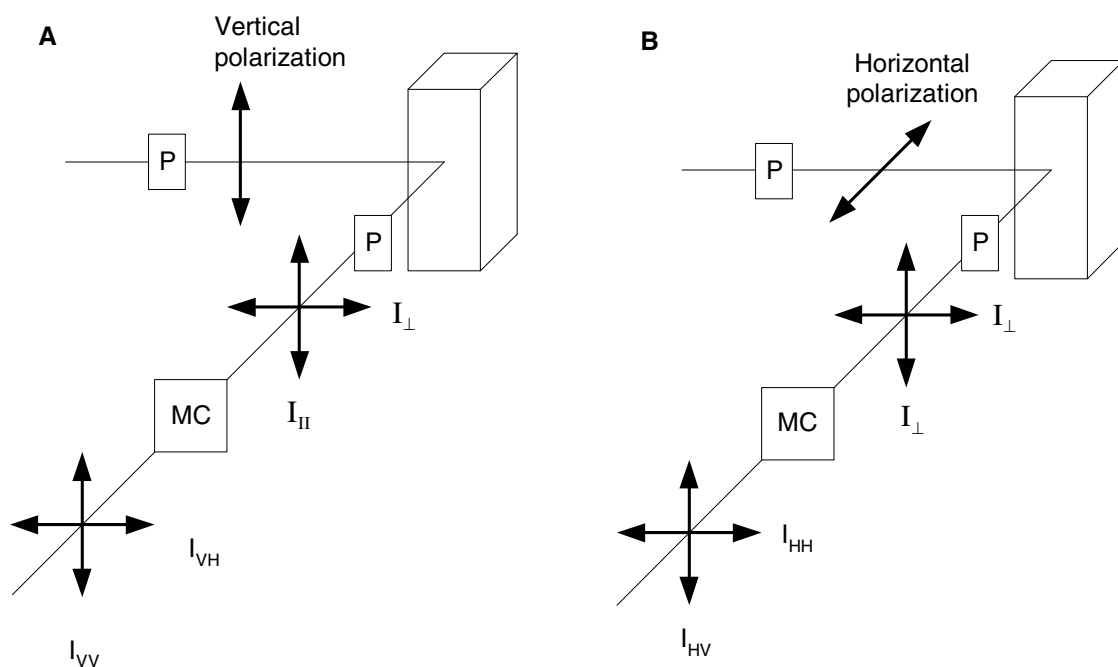


Figure 16: Measurement of steady-state fluorescence anisotropy with the L-format instrumentation. Vertical (A) or horizontal (B) polarized light excites the sample and the parallel and perpendicular emissions are detected through the monochromator (MC) by a photomultiplier.

1.5.1.4 Time Resolved Fluorescence Methods

1.5.1.4.1 Time resolved emission intensity/lifetime

The fluorescence lifetime represents the average time the fluorophore is in the excited state following excitation, before returning to the ground state. It is the time required for the intensity to decay to $\frac{1}{e}$ of its initial value. There are two methods for measuring fluorescence lifetimes: time domain and frequency domain [163]. In time domain, measurements of lifetime are determined from the slope of the fluorescence intensity ($\log I(t)$) *versus* time. For frequency domain or phase-modulation, the sample is excited by amplitude-modulated excitation light and the shift in phase and modulation of the fluorescence emission is related to the fluorescence lifetime. In the present study time domain measurement of the fluorescence lifetimes was employed.

For time domain studies [163, 207-210], the fluorophores are excited using a narrow pulse of light. The population of excited state fluorophores decays according to the equation:

$$\frac{dN(t)}{dt} = -(\Gamma + k_{nr})N(t) \quad (27)$$

where $N(t)$ is the number of fluorophores in the excited state, Γ is the emissive radiative rate and k_{nr} is the non-radiative decay rate. Integration of this equation with $N(t) = N_0$ at $t = 0$ yields the decay of the fluorescence intensity, which is proportional to the number of excited molecules in the excited state:

$$I(t) = I_0 \exp(-t / \tau) \quad (28)$$

where I_0 is the intensity at time (t) zero and τ is the lifetime of the fluorophore ($\frac{1}{\Gamma + k_{nr}}$).

Protein molecules generally show multi-exponential fluorescence decays and the decay expression is rewritten as:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \quad (29)$$

where α_i is the pre-exponential factor associated with fluorescence lifetime component, τ_i . The average fluorescence lifetime, $\bar{\tau}$, for a multi-exponential decay, is given by the equation:

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \quad (29a)$$

Measurement of fluorescence lifetime is useful for protein folding studies. In addition to information about the excited state photophysics of the fluorophore, the fluorescence lifetime may also be sensitive to conformational transitions [201] and be able to resolve the contributions of a mixture of global states [173].

1.5.1.4.2 Time Resolved Emission Anisotropy

For time resolved emission anisotropy (TREA) studies, following excitation, polarized emission decay intensities ($I_{VV}(t)$ and $I_{VH}(t)$) are recorded. The decay of emission anisotropy ($r(t)$) can be influenced by the overall rotation of the macromolecule and by internal motions of the fluorophores [207]. There are three internal motions:

- 1) rotation of the fluorophore about the bond linking it to the macromolecule;
- 2) rotation of a small segment of the molecule close to the fluorophore; and
- 3) rotation of a domain as a unit about a hinge point.

The anisotropy decay is defined as:

$$r(t) = \sum_j^n \beta_j e^{-t/\phi_j} \quad (30)$$

where β_j is the partial anisotropy associated with the rotational correlation time, ϕ_j . The number of j terms depends on the measured rotational motions of the protein and fluorophore [207]. The limiting anisotropy, r_0 , is defined as:

$$r_0 = \sum_j^n \beta_j \quad (31)$$

and is related to the angle between the absorption and emission dipoles.

A comparison of the steady state anisotropy can be determined from time resolved parameters by the equation [171]:

$$r(t)_{ss} = \frac{r_0 \sum_i \sum_j \alpha_i \beta_j \left(\frac{1}{\tau_i} + \frac{1}{\phi_j} \right)^{-1}}{\sum_i \alpha_i \tau_i} \quad (33)$$

From Equation 33, the steady state emission anisotropy values can provide information about the averaged rotational motions of a macromolecule. However, time resolved measurements are able to differentiate between global, segmental or local fluorophore rotations.

1.5.2 Gel Electrophoresis

Electrophoresis is a major analytical technique for protein analysis [211-213]. It is used to separate proteins on an inert gel matrix using an electric field. Most globular proteins are generally charged molecules with pI values around 4 to 5 [214]. The charges are derived from ionic amino acids: basic (arginine, lysine and histidine) and acidic residues (glutamic and aspartic acids).

Electrophoresis follows Ohm's law (Equation 35) where V is voltage, I is the current and R is the resistance through the gel:

$$V = I * R \quad (35)$$

In an electric field, E (V/cm), the force (F) acts on a protein depending on its net charge, z , according to the function:

$$F = Ez \quad (36)$$

So, as the net charge (z) increases, the greater the electrostatic force (F). The migration velocity (v) of a protein in the unit electrical field ($E = 1$ V/cm) is defined as:

$$v(cm/h) = E(V/cm) * u(cm^2/Vh) \quad (37)$$

where u , the mobility, is equal to the net charge of the protein (q) divided by the friction coefficient (f). The rate of migration depends not only on the electrical field but also on

characteristics of the protein (net charge, size and shape) and indirectly, higher current can affect mobility and migration velocity by decreasing the viscosity of the medium, so it is essential to dissipate the heat generated.

For protein electrophoresis, the most commonly used medium is polyacrylamide. The gel is prepared by the polymerization of acrylamide with a cross-linker N, N'-methylenebisacrylamide (bisacrylamide) to form a three dimensional network [212]. In general, discontinuous buffer systems are used and they utilize two gel components: stacking and separating gels [215]. The stacking gel concentrates the lower ionic strength protein sample *via* a voltage gradient. There is no significant sieving in this gel because of the large pores (2.5 - 4% acrylamide). In the separating gel, the pores are smaller (7.5 – 20% acrylamide) so as the protein sample migrates, the molecules are sieved. An acrylamide gradient is often used in the separating gel.

1.5.2.1 Native Polyacrylamide Gel Electrophoresis (PAGE)

The electrophoretic mobility of a protein depends on its charge, shape and molecular weight. In a native PAGE, proteins migrate based on hydrodynamic size, shape and electrostatic charge. It is often used to separate soluble proteins [212]. Native PAGE does not denature proteins, so they retain their biological properties. It is a frequently used analytical technique to monitor protein purity.

1.5.2.2 Theory of SDS-PAGE

SDS is a negatively charged surfactant (Figure 1) and reduces oligomeric proteins into monomers [30]. Proteins bind a maximum of 1.4 g SDS per g protein [29], and form

a complex [38]. In 4% (0.14M) SDS, the SDS-protein complexes all have a very high (and similar) charge/mass ratio and similar conformations that differ only in size [212, 213]. Thus, separations in SDS-PAGE are due to the hydrodynamic size of proteins only. SDS-PAGE, the method of choice for determination of molecular weights [211], is based on the migration distance between the protein and the bromophenol blue tracking dye, which is linearly proportional to the log molecular weight (MW). By running standard proteins to produce a calibration curve, the unknown molecular weight for the proteins may be determined. Furthermore, the effect of SDS on the protein oligomeric state is easily visualized by this technique. In the case of a dimeric protein, it is quite easy to monitor dissociation by electrophoresis in the presence of SDS.

1.5.2.3 Transverse Urea Gradient Gel Electrophoresis (TUGGE)

Transverse gradients provide an elegant approach for studying protein conformational changes [215, 216]. The electrophoretic mobility of a protein is examined using a transverse gradient of urea incorporated within the gel and perpendicular to the direction of electrophoresis. Thus, the protein will migrate through a gel of varying urea concentrations.

Transverse urea gradient gels are only slightly more complicated than native and SDS-PAGE to prepare and use small amounts of protein. The unfolding of a protein is monitored by the decrease in electrophoretic mobility due to the larger hydrodynamic volume as the protein conformation changes from compact globule to unfolded random chain polypeptide. The protein applied to the gel is native, so the unfolding transitions occur during the electrophoretic run time. A key advantage of UGGE is the ability to

directly monitor the folding transitions of proteins and this technique can be used to obtain protein conformational information (dimer, monomer) including quantification of the amount and identity of the molecules in solution.

Furthermore, thermodynamic information and the kinetics of unfolding can also be determined. Thermodynamic stability of native proteins is monitored relative to the unfolded protein. In urea gradient gels, this is determined from the average mobility of the native and unfolded states only if the protein undergoes a rapid two-state transition. The kinetics of unfolding is determined from the continuity of the band as the protein transitions from the native to unfolded state; if folding is rapid compared with the time of the electrophoretic run, then there is a sharp continuous transition band; and if folding is slower than the electrophoresis run, the band will be discontinuous or smeared out.

Since urea is a neutral molecule, the migration of a protein through UGGE depends on hydrodynamic size and electrostatic charge. UGGE is able to capture and quantitatively identify transient kinetic intermediates present during denaturation and renaturation that are undetected in equilibrium experiments [215]. UGGE has been used to monitor protein unfolding [218] and the dissociation of trimers [217] and dimers [87].

1.5.3 Interaction of small molecules with proteins

1.5.3.1 Enzyme Inhibition

At times, when the denaturants urea and GuHCl are used to induce protein denaturation, it is unclear whether the decrease in activity is due to the effect of a conformational change [50, 75, 82, 91, 219, 220] or is simply the result of substrate inhibition, especially if enzyme turnover involves an amine substrate or product. In such cases, it is important to perform substrate inhibition studies to rule out the possibility that the inhibition is being caused by the denaturant.

Substrates (S) and inhibitors (I) bind to enzymes (E) in a specific manner to form a complex [221]. A substrate binds to the enzyme and after turnover, products leave the enzyme as it was prior to substrate binding (Equation 38):



For a one substrate, one enzyme process, the K_m or Michaelis constant, can be determined from velocity (v) measurements. As the concentration of substrate ([S]) increases, there is a hyperbolic increase in the activity according to the Michaelis-Menten equation (assuming $[S] \gg [E]$):

$$v = \frac{V_{\max} [S]}{K_m + [S]} \quad (39)$$

where $V_{\max}$ is the maximum velocity and K_m is the Michaelis constant, which is the substrate concentration at $\frac{1}{2} V_{\max}$. The data may be linearized according to Lineweaver-Burk double reciprocal plots of $1/[S]$ vs $1/v$ by the equation:

$$\frac{1}{v} = \frac{K_m}{V_{\max} [S]} + \frac{1}{V_{\max}} \quad (40)$$

where the slope is $K_m/V_{\max}$ and the y intercept is $1/V_{\max}$.

There are many different ways in which a protein can interact with an inhibitor, and the influence of inhibitors can also be monitored by changes in activity or velocity (v) of the enzymatic reaction. In general there are five different types of inhibition that occur [221]: competitive, noncompetitive, irreversible, uncompetitive and a linear-mixed type of the previous four inhibitions.

In competitive inhibition, both the substrate and inhibitor (I) compete for the binding site on the enzyme (E):



Two enzyme complexes are formed: ES (Equation 38) and EI (Equation 41). The $V_{\max}$ for the enzyme remains unchanged but the K_m increases by a factor of $1 + \frac{[I]}{K_i}$ with inhibition constant, K_i . The Lineweaver-Burk plot may now be re-expressed as:

$$\frac{1}{v} = \frac{K_m}{V_{\max} [S]} \left(1 + \frac{[I]}{K_i} \right) + \frac{1}{V_{\max}} \quad (42)$$

$$slope = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \quad (43)$$

In the presence of a competitive inhibitor, a plot of the slope calculated from the Lineweaver-Burk double reciprocal plot (40) *versus* [I] results in an intercept of $K_m/V_{\max}$ and a slope of $K_m K_i/V_{\max}$. Therefore, the intercept divided by the slope provides estimates of K_i .

1.5.3.2 Non-covalent binding of ligands

During the unfolding of a protein, ligands may demonstrate varying affinities for possible conformational intermediates. This is important for measuring the binding of the hydrophobic site sensitive fluorescence probes ANS and bis-ANS (Section 1.5.1.1.2.2).

In general, the number of ligands bound (n) and K_A (association constant) are determined by Equation 44 [222-224]:

$$\bar{v} = \frac{nK_A [L_f]}{1 + K_A [L_f]} \quad (44)$$

$$\bar{v} = \frac{[L_b]}{[P]} \quad (45)$$

where $\bar{v}$ is the concentrations of bound ligand (L_b) per total protein concentration (P) (Equation 45), and the concentration of free ligands in solution (L_f) is equal to the total ligand concentration minus bound ligand ($L_f = L_T - L_b$). However, for conditions where the concentration of ligand is much greater than that of the protein ($L > P$), then the free ligand (L_f) is equivalent to the total ligand concentration (L_T) since ligand bound (L_b) is small.

From fluorescence titrations, the fractional occupancy of the binding sites (θ) can be measured [224] and is equivalent to $\frac{\bar{v}}{n}$:

$$\theta = \frac{F - F_0}{F_{\max} - F_0} = \frac{\Delta F}{\Delta F_{\max}} = \frac{\bar{v}}{n} \quad (46)$$

where F is the observed fluorescence and $F_{\max}$ is maximum fluorescence intensity when all protein sites are saturated with the fluorophore ligands, which are corrected for the fluorescence contributions from the ligands in the absence of protein (F_0). Rearrangement of Equations 45 and 46 provides Equation 47 where plots of $\Delta F/\Delta F_{\max}$ give the association constant, K_A ($1/K_D$):

$$\frac{\bar{v}}{n} = \frac{K_A [L_f]}{1 + K_A [L_f]} = \theta \quad (47)$$

This approach is especially useful for comparing different protein conformations [16, 20, 22, 225] since formation of a possible molten globule intermediate exposes hydrophobic sites during the unfolding and/or refolding of a protein [14]. In addition, the

native state of proteins exhibit differences in the exposure of hydrophobic patches as shown in Table 7. Analysis of Equations 45 or 47 for ANS and bis-ANS binding shows low binding affinities (high K_D) to some proteins (e.g. alkaline phosphatase [20]) and high affinities (low K_D) for others (e.g. BSA [21]). Similarly, the number of probes bound to the protein can vary from 1 (ovalbumin [21]) to 54 (bacteriorhodopsin [21]).

Table 7
Binding of hydrophobic probes to different proteins.

Protein	n ANS	K _D ANS (μ M)	n bis- ANS	K _D bis- ANS (μ M)	Reference
α -crystallin			40	1.15	[22]
α -lactalbumin			2.6 $\pm$ 0.6	13.3	[226]
β -lactalbumin			5		[225]
β -lactamase	2	40			[16]
Alkaline phosphatase		165.4 $\pm$ 29.8		5.4 $\pm$ 0.9	[20]
Bacterio-rhodopsin	54	1.1			[21]
BSA	10	1.22			[21]
Creatine kinase	1	310			[227]
DnaK			1	7	[228]
Factor VIII				0.2	[229]
IFABP ^a		16.0 $\pm$ 1.7		8.6 $\pm$ 2.9	[230]
Lysozyme	3	13			[21]
Manganese peroxidase				1.6	[231]
Ovalbumin	1	1.32			[21]
Papain	21				[232]
PPAO ^b	1	5			[233]
Protease omega	36				[232]
Rec-A	3.5	34	>40		[234]
RNAase	1	80			[21]

^a Intestinal fatty acid binding protein

^b Pig plasma amine oxidase

1.5.4 High performance liquid chromatography (HPLC)

HPLC is a frequently used separation technique for both analytical and preparative purposes [235, 236]. Size-exclusion chromatography HPLC (SEC-HPLC) elutes macromolecules with decreasing molecular size and shape [236]. Therefore, this is a good technique to monitor protein interactions like the dissociation of a macromolecule or unfolding. The limit of elution volume is between the void (V_0) and total (V_t) volumes of the column.

The elution volume (V_e) is calculated from the retention time (R_t) and flow rate (R_f) by the equation:

$$V_e = (R_t) * (R_f) \quad (34)$$

The Stokes radii (R_s) and molecular weights (MW) are determined by linear evaluation of V_e and standard R_s or standard MW.

SEC-HPLC is used to monitor not only the formation (e.g. cytomegalovirus protease [237], 4-oxalocrotonate tautomerase [238]) and dissociation of dimers (e.g. prostatic acid phosphatase [80]), but also the presence of intermediate conformations. Some intermediate conformations during dissociation to a monomer or unfolded monomer include aggregates ($D_2 \rightarrow A \rightarrow 2M^U$) (e.g. triosephosphate isomerase [47]), monomers ($D_2 \rightarrow 2M \rightarrow 2M^U$) (e.g. glutathione transferase [79]) or unfolded dimers ($D_2 \rightarrow D_2^U \rightarrow 2M^U$) (e.g. glutathione reductase [239]).

Chapter 2: Materials and Methods

2.1 Materials

Adult bovine serum (B-2771), aniline hydrochloride (A-8524; 129.59g/mol) and ω -aminohexyl agarose (A-6017) were obtained from Sigma Aldrich Company (Missouri). Urea (U-0631; 60.06g/mol), SDS (L-6026; 288.38g/mol) and GuHCl (G-7153; 95.53g/mol) were SigmaUltra grade (Sigma Aldrich, MO). Pyrene maleimide (P-28; 297.31g/mol), bis-ANS (B-153; 672.85g/mol) and ANS (A-47; 299.34g/mol) were obtained from Molecular Probes Inc. (Oregon). All other materials were reagent grade and used without further purification.

The buffer used in all experiments was 0.02M Hepes with 0.1M NaCl, pH 7.5 filtered using a 0.45 μ m nylon filter membrane (Sigma Aldrich, MO) unless otherwise noted.

2.2 Methods

2.2.1 Protein Activity Assay

The activity of BPAO was assayed spectrophotometrically as described by Turini *et al* [153]. A Hewlett Packard Diode Array Spectrometer (8452A), with a thermostatically regulated sample holder, was used to follow the BPAO catalyzed production of benzaldehyde from benzylamine (107.15g/mol) at 250nm for 5 minutes. The extinction coefficient for benzaldehyde at 250nm is 12500M⁻¹cm⁻¹ [146]. One unit of BPAO activity (U) is defined as the production of 1 μ mol of benzaldehyde/min at 25°C [160]. After purification, the concentration of BPAO was determined from the BCA

assay [240]. Subsequent protein concentrations were determined using an extinction coefficient ($\epsilon_{280}^{1\%}$) of 20.8 [146]. The specific activity (SA) of BPAO was calculated using:

$$SA_{\text{BPAO}} = \text{Units of activity} / \text{mg protein} \quad (48)$$

2.2.2 Protein Isolation and Purification

BPAO was purified according to the method of Vianello *et al* [146]. The protein was first precipitated from bovine plasma using 35% (w/v) and 55% (w/v) ammonium sulfate in the supernatant and pellet, respectively. The 55% (w/v) pellet was re-dissolved in deionized water and dialyzed overnight using 8 kD molecular weight cut-off dialysis tubing (5/8inch width; Arthur H. Thomas Co, PA) with at least three changes of the deionized water to remove any remaining ammonium sulfate. Affinity chromatography was then performed using a ω -aminohexyl agarose gel. The column (2.3 x 20.5cm) was washed and equilibrated using 8 column volumes of the standard Hepes/NaCl buffer. The protein was either loaded onto the affinity column or pre-equilibrated with the ω -aminohexyl agarose gel prior to packing a column, and then washed with Hepes/NaCl buffer until $A_{280} < 0.05$. BPAO was eluted from the column with two column volumes of 0.2M aniline hydrochloride in Hepes/NaCl buffer, pH 7.5. The resultant protein-aniline eluant was concentrated using a Centriprep YM-30 centrifugal filter unit (Millipore, MA) followed by desalting on a BioRad Econo-Pac[®] 10DG disposable chromatography column (BioRad, CA) at least 3 times to ensure that the aniline hydrochloride was removed from the protein solution. The BPAO concentration was determined using a

standard BCA assay [240]. Table 8 summarizes typical protein purification statistics and shows the %yield and fold purification of BPAO at different steps along the procedure. The specific activity values obtained were comparable with those previously obtained [146].

The purity and homogeneity of BPAO used for the spectroscopic measurements was assessed using polyacrylamide gel electrophoresis (PAGE), gel-filtration and enzyme kinetics (see Methods Section 2.2.7, 2.2.6 and 2.2.1; Results Section 3.1).

Table 8:
Purification Table for Bovine Plasma Amine Oxidase.

Purification Step	Total Protein (mg)	Total Units (U)	Specific Activity (U/mg)	¹ Yield (%)	² Fold purified
Plasma	6,436	307,641	47.8	100	1.00
35% (NH ₄) ₂ SO ₄ supernatant	4,574.1	290,913	63.6	95	1.33
55% (NH ₄) ₂ SO ₄ pellet	1,005.5	241,521	240.2	79	5.03
Affinity chromatography, dialysis and ultra-filtration	31.5	81,333	2,582	26	54.02

¹The yield is calculated from the total units of activity.

²The fold purified is determined from the specific activity.

2.2.3 Unfolding and Refolding Conditions

The unfolding of dimeric BPAO was achieved using either guanidine hydrochloride (GuHCl), urea or sodium dodecyl sulfate (SDS). Stock solutions of these protein denaturants were prepared in 0.02M Hepes/0.1M NaCl, pH 7.5. The pH of the urea and GuHCl solutions was adjusted to 7.5 using sodium hydroxide. GuHCl samples were stable for at least one month after preparation whereas urea samples were prepared daily to minimize hydrolysis. The refractive index of these solutions, determined by a Bausch & Lomb ABBE3-L refractometer equipped with a Haake FE water bath at 25°C, was used to determine the actual concentrations of GuHCl and urea. The difference in refractive index values (ΔN) for solutions of the perturbant and pure buffer may be related to the actual concentrations of GuHCl (Equation 49) and urea (Equation 50) by the equations [241]:

$$[GuHCl] = 117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3 \quad (49)$$

$$[Urea] = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3 \quad (50)$$

A stock solution of SDS (4% (w/v) or 0.138mM) in buffer was prepared above the critical micelle concentration (cmc) for SDS. Generally, the cmc decreases with increasing ionic strength: it is ~8mM in water [29, 242] but ~1mM at 0.12M ionic strength [29]. In these studies, the cmc was not determined in 0.02M Hepes/0.1M NaCl, pH 7.5 but is expected to be ~1mM for an ionic strength of 0.12M [29], which is

comparable to the buffer used in these studies. The maximum SDS concentration used in these studies was 14mM, which is well above the cmc value.

For unfolding studies, BPAO was incubated in varying concentrations of GuHCl (0-6M) or SDS (0-0.014M) overnight, in the dark and at room temperature while urea (0-8M) was incubated in the dark at room temperature for 8 hours. Wavelength shifts in the tryptophan emission spectrum of BPAO observed upon unfolding with 5M GuHCl or 8M urea were achieved within minutes (data not shown). However, to ensure that conformational equilibrium was achieved, 8 hours (urea) to overnight (GuHCl and SDS) incubation of the protein in the denaturants was chosen as the standard unfolding conditions.

For protein refolding studies, BPAO was first pre-incubated in 5M GuHCl or 8M urea for 3 hours to ensure complete protein unfolding. The denaturant was then diluted to the desired final concentration, using varying concentration ratios of Hepes/NaCl buffer and stock solutions of denaturant, so that a constant protein concentration was used for all samples. The perturbed protein was then left overnight at room temperature and in the dark prior to spectroscopic analysis.

2.2.4 Labeling of BPAO with Pyrene Maleimide

Labeling of free SH groups on BPAO was achieved using the fluorescence probe pyrene maleimide. Analysis of the surface cysteines in BPAO (PDB: 1TU5 [113]) by Rasmol 2.7.2.1 [139] shows the presence of only one free cysteine residue (C94) per monomer. A pyrene maleimide stock solution ($1 \times 10^{-3}\text{M}$) was prepared in DMSO and the concentration was determined using the extinction coefficient, $\epsilon_{338} = 40,000\text{M}^{-1}\text{cm}^{-1}$

[244]. BPAO in standard Hepes/NaCl buffer, pH 7.5 (8×10^{-6} M) was incubated (1:10 or 1:100 protein:fluorophore mole ratio) at room temperature, overnight and in the dark with pyrene maleimide. Unbound pyrene maleimide was separated from protein bound fluorophore using a BioRad Econo-Pac[®] 10DG disposable chromatography column (BioRad, CA) by elution with standard Hepes/NaCl buffer. The concentration of bound pyrene maleimide was calculated spectrophotometrically, since the extinction coefficient in DMSO and Hepes buffer were determined to be equivalent (data not shown). The concentration of BPAO was estimated using the Micro BCA assay [240]. From a standard BSA calibration curve, the concentration of BPAO (relative to BSA) was determined from:

$$A_{562} = 0.02938[\text{Protein}] \quad (51)$$

The average molar labeling ratio of pyrene maleimide to BPAO from three different labeling trials was found to be 2.00 ± 0.17 , which suggests that each subunit is labeled with one pyrene moiety. This is consistent with one free cysteine group (C94) per monomer on the surface [113]. Interestingly, if the incubation time for the labeling process is reduced from overnight to the manufacturer recommended three hours, a labeling ratio of only one pyrene per dimeric protein was determined. This suggests that the relative reactivity of each exposed –SH groups to pyrene maleimide may be different. This has also been observed for enzyme I in the phosphoenolpyruvate:glucose phototransferase system (PTS) [245] and translin [181].

2.2.5 Spectroscopic Methods for Studying Protein Unfolding

2.2.5.1 Steady State Fluorescence Studies

Steady state fluorescence measurements were performed using a Spex Fluorolog Tau 2 fluorimeter (SPEX-Horiba Instruments, Inc., New Jersey) equipped with a 4-sample thermostatted cuvette holder. All measurements were performed at 25°C, which was achieved using a VWR Scientific 1167 circulating water bath equipped with a computer controlled temperature feedback from the sample chamber. The excitation and emission slit widths for all experiments were 3 nm and 2.5 nm, respectively, unless stated otherwise. Using standard right-angle emission optics, fluorescence intensity measurements were recorded using the photon counting mode and were corrected for any fluctuations of the 450-Watt Xenon lamp source by deflecting a portion of the excitation signal on to a separate photodiode. The measured intensity signals are thus reported in the ratio mode (S/R) as counts per second (S) measured on the emission photomultiplier over the photodiode current (R). Measurements were performed using quartz fluorescence cuvettes with path-lengths of 10mm or 3mm. For all fluorescence studies, samples were adjusted to provide an absorbance value at the excitation wavelength of less than 0.1. Under such conditions, possible inner filter effects [246] are minimized and the fluorescence signal is proportional to concentration.

For steady state fluorescence anisotropy measurements, Glan-Thompson UV polarizers were inserted into the excitation and emission optical paths. Polarized fluorescence intensities were recorded for all four possible excitation and emission polarizer configurations (I_{VV} , I_{VH} , I_{HH} , I_{HV}) using a photon counting integration time of 10 s for each orientation. The emission anisotropy ($\langle r \rangle$) was calculated according to:

$$\langle r \rangle = \frac{I_{VV} - GI_{VH}}{I_{VV} + G.2I_{VH}} \quad (22)$$

where the first subscript reflects the orientation of the excitation polarizer, and the second subscript that of the emission polarizer. G is the grating factor which compares the instrument emission detection sensitivity to vertical and horizontal excitation light [205]:

$$G = \frac{I_{VH}}{I_{HH}} \quad (23)$$

The denominator of Equation 22 is equivalent to the total fluorescence intensity (Equation 24):

$$\text{Total Intensity} = I_{VV} + G.2I_{VH} \quad (24)$$

which was used to monitor intensity changes (and therefore possible lifetime affects (Equation 19)) that occur during the unfolding process.

Polarized intensities were collected for both the protein sample of interest and the control solution. Polarized intensity values for the blank were then subtracted from the corresponding polarized intensity value for the sample (e.g. $I_{VV} = I_{VV}(\text{sample}) - I_{VV}(\text{blank})$) before calculation of the emission anisotropy according to Equation 22. Reported anisotropy values represent the mean of at least three separate measurements. All data were collected at 25°C.

Tryptophan Studies: Tryptophan residues in BPAO were excited at 295nm and the emission spectra were monitored from 315 to 440nm. Samples were measured using standard 'H' cuvettes with a 10mm excitation and 2mm emission pathlength. The center of spectral mass (CSM) was calculated from the fluorescence emission intensity (F) at a particular wavelength (λ) in the range 315 – 440 nm by Equation 17:

$$CSM = \frac{\sum_i \lambda_i F_i}{\sum_i F_i} \quad (17)$$

The integrated intensity (II) was determined from the fluorescence emission intensity (F) in the range 315 to 440nm by the equation:

$$II = \sum_i F(\lambda_i) \quad (53)$$

Emission anisotropy values for tryptophan were collected using 295nm and 350nm, as the excitation and emission wavelengths, respectively, using 10mm pathlength cuvettes.

Pyrene Maleimide: Fluorescence studies of PM labeled BPAO (PM-BPAO) were achieved using excitation and emission wavelengths of 344nm and 377nm, respectively, with a 3mm excitation and emission path lengths cuvette.

Bis-ANS: Bis-ANS and ANS concentrations were determined from their extinction coefficients ($\epsilon_{395\text{nm}} = 23000 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{372\text{nm}} = 7900 \text{ M}^{-1}\text{cm}^{-1}$, respectively, in methanol) [244]. The excitation and emission wavelengths used were 395nm and 500nm, respectively.

Binding of bis-ANS to BPAO were followed by measuring the increase in fluorescence intensity of the fluorophore when associated with the protein. For the titration of BPAO with the bis-ANS or ANS probe ($5 \times 10^{-4}\text{M}$), the excitation path length was 2mm and the emission path length was 10mm. For the titration of a fixed probe concentration with varying BPAO concentrations, the excitation and emission pathlengths were 10mm and 2mm, respectively. Control samples were recorded and used to correct for dilution effects on the measured fluorescence intensities due to increased sample volumes (up to 5%) as a result of the titrations.

For the titration or equilibration of protein first denatured in GuHCl or urea with bis-ANS, BPAO solutions were unfolded in the denaturant (Section 2.2.3) prior to addition of the fluorophore. The binding occurred within minutes (data not shown), but was allowed to equilibrate for 30mins before spectroscopic studies were performed. The effect of denaturant on bis-ANS fluorescence intensities was monitored by a control experiment and was found to be <1%.

2.2.5.1.1 Non-covalent Fluorescent Probe Binding Analysis

The binding constant for the interaction of ANS and bis-ANS ligands with BPAO can be obtained from fluorescence intensity measurements [222-224]. Given that ANS and bis-ANS are virtually non-fluorescent when free in solution, the increase in fluorescence intensity on binding to a macromolecule measures the fractional occupancy of binding sites (θ , Equation 46) from the observed fluorescence (F) and maximum fluorescence intensity, determined when all protein binding sites are saturated with the fluorophore ligands ($F_{\max}$). All intensities are first corrected for the fluorescence contributions from the ligands in the absence of protein (F_0):

$$\theta = \frac{F - F_0}{F_{\max} - F_0} = \frac{\Delta F}{\Delta F_{\max}} \quad (46)$$

The concentration of bound ligand (L_b) per total protein concentration (P), $\bar{v}$ (Equation 45), together with the free ligand concentration (L_f), can be used to calculate the number of ligands bound (n) and K_A (the association constant) by Equation 44.

$$\bar{v} = \frac{[L_b]}{[P]} \quad (45)$$

$$\bar{v} = \frac{nK_A[L_f]}{1 + K_A[L_f]} \quad (44)$$

The fractional occupancy of the binding sites (θ) is equivalent to $\frac{\bar{v}}{n}$, and rearrangement of Equation 44 gives Equation 54:

$$\theta = \frac{K_A [L_f]}{1 + K_A [L_f]} \quad (54)$$

Since the concentration of ligand is much greater than that of the protein, the free ligand (L_f) is equivalent to the total ligand concentration. Linear analysis by the double reciprocal plot of Equation 54 and substitution of $1/K_D$ (the dissociation constant) for K_A gives:

$$\frac{1}{\theta} = 1 + \frac{K_D}{[L_f]} \quad (55)$$

A plot of $1/\theta$ *versus* $1/[L_f]$ gives a slope equivalent to the K_D .

For fluorescence studies, it is not always possible to add enough ligand concentration to the protein to achieve $F_{\max}$ due to inner filter effects. In such cases, $\Delta F_{\max}$ is determined from the titration of the ligand by the protein [223]. From a double reciprocal plot of $1/\Delta F$ *versus* $1/[P]$, at the point of infinite protein concentration, a value of $\Delta F_{\max}$ may be obtained. For bis-ANS, the $\Delta F_{\max}$ values were calculated by both the titration of BPAO and by the hyperbolic maximum of the titration curve using 1.01×10^{-6} M bis-ANS were determined to be 3.29×10^7 and 3.38×10^7 , respectively. For ANS,

$\Delta F_{\max}$ was determined only from the hyperbolic maximum by the titration of ANS with BPAO ($\Delta F_{\max} = 6.1 \times 10^6$).

2.2.5.2 Time Resolved Fluorescence Studies

Time resolved fluorescence measurements were performed using the time-correlated single photon counting technique using a synchronously pumped, frequency doubled, cavity pumped dye laser (repetition rate 4 MHz, pulse width 2 ps, average UV power < 200 μ W) as the excitation source. The pump laser used was a 10W Spectra Physics water-cooled argon/ion laser.

Fluorescence lifetime decays for PM-BPAO samples, excited at 335 nm, were collected under ‘magic angle’ (54.7° to the vertical for vertically polarized laser light [208]) emission polarization conditions with the emission monochromator set at either 400nm or 420 nm. The channel width was 173 ps, and the data were collected over 512 channels using a multichannel analyzer (Ortec, TN). The BPAO concentration was $8.25 \pm 1.10 \times 10^{-5}$ M for all protein/GuHCl samples studied. Samples were measured using 10mm path length quartz cuvettes at 28°C.

For time-resolved fluorescence emission anisotropy (TREA) measurements, a rotating polarizer was used on the emission optical train to provide parallel (I_{VV}) or perpendicular (I_{VH}) polarized emissions with respect to the vertically polarized laser excitation. Polarized decay data were collected to 10,000 counts in the peak of the I_{VV} decay profile. Four sets of each of the two polarized intensity decays were collected and summed to provide decay profiles with a sufficient number of peak counts for an appropriate statistical analysis.

For all time-resolved fluorescence experiments, a reference lamp decay profile was collected contemporaneously with the data using a sample of Ludox (a non-fluorescent colloidal silica) for deconvolution of the lamp profile. In addition, the photomultiplier color shift effect also needed for the convolution analysis, together with the performance of the instrument, was accessed using a well characterized mono-exponential decaying standard (9-cyanoanthracene in ethanol, with a fluorescence lifetime, $\tau = 11.85 \pm 0.03$ ns [247]).

2.2.5.2.1 Data Analysis

Time resolved data were analyzed using a non-linear least squares fit of the experimental data to a theoretical convolution function [166, 181]. In all cases, the best fit was estimated from evaluation of the weighted residuals and the reduced chi-squared (χ^2) value.

Measured fluorescence intensity decays were analyzed using a multiexponential function together with an independent parameter, α_s , which represents any contributions from Rayleigh scattering:

$$I(t) = \sum_i^n \alpha_i e^{-\frac{t}{\tau_i}} + \alpha_s \delta(t) \quad (56)$$

The recovered relative amplitudes (α_i) and fluorescence lifetimes (τ_i) were represented as short, medium and long decay components according to the lifetimes. For α_{short} , “short” implies all species with the lowest lifetime ($\tau \sim 0.2$ - 0.5 ns; Table 12).

The decay of the fluorescence emission anisotropy is defined as:

$$r(t) = \sum_j^n \beta_j e^{-t/\phi_j} \quad (30)$$

Experimentally the components, β_j and ϕ_j , were obtained using the “sum and difference method”:

$$r(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + G \cdot 2I_{VH}(t)} = \frac{D(t)}{S(t)} \quad (32)$$

where G is the ratio of sensitivities of the detector for vertically and horizontally polarized light (Equation 9). $D(t)$ represents the decay of the difference in polarized decay components and $S(t)$ is the decay of the total (or sum) of emission intensities (Equation 57). Given that:

$$S(t) = I_{VV}(t) + 2GI_{VH}(t) = \sum_i^n \alpha_i e^{-t/\tau_i} \quad (57)$$

and the decay of the difference in polarized intensities, $D(t)$, from Equation 32 is:

$$D(t) = r(t) \cdot S(t) \quad (58)$$

Then:

$$D(t) = r(t) \cdot \sum_i \alpha_i e^{-t/\tau_i} \quad (59)$$

and with substitution of Equation 30 for $r(t)$, gives:

$$D(t) = \sum_j \beta_j e^{-t/\phi_j} \cdot \sum_i \alpha_i e^{-t/\tau_i} \quad (60)$$

The values for α_i and τ_i were first extracted from the decay of the sum of polarized intensities (Equation 57). The resolved values were then fixed and a difference analysis was performed by a non-linear iterative fitting of Equation 60 to the experimental difference decay profile (Equation 32) to obtain values for β_j and ϕ_j . The best fits were resolved into two or three rotational components.

Short, medium and long decay components are classified according to the rotational correlation time (ϕ_j). The fraction of each component contribution to the decay profile, f_{β_j, ϕ_j} , was determined from the relative contribution of each component divided by the sum of the contributions:

$$f_{\beta_j, \phi_j} = \frac{\beta_j \phi_j}{\sum_j \beta_j \phi_j} \quad (61)$$

where “short” implies all species with $\phi \sim 0.4\text{-}1.5$ ns (Table 13). The rotational correlational times obtained (ϕ_j) were corrected for viscosity changes on addition of GuHCl (Table 9) [248]:

$$\phi_{corr} = \phi_j \left(\frac{\eta}{\eta_0} \right) \quad (62)$$

where η is the relative viscosity of the GuHCl solution compared to water (η_0) at 25°C.

Table 9:
Relative viscosities of GuHCl solutions at 25°C

[GuHCl] (M)	η/η_0 ^a
1	1.04
2.5	1.13
3.5	1.20
5	1.41

^a η/η_0 is the relative viscosity of the GuHCl solution compared to water at 25°C.
Taken from Kawahara K and Tanford C (1966) Viscosity and density of aqueous solutions of urea and guanidine hydrochloride. *J Biol Chem.* 241(13): 3228-3232 [248]

2.2.6 HPLC Analysis of Protein Conformation

2.2.6.1 Sample preparation and Column Characterization

Size-exclusion HPLC experiments were performed using a BioSep S3000 gel filtration column (300 x 4.60mm; Pharmacia, CA). The Binary LC pump 250 (Perkin Elmer), the Diode Array detector 235C (Perkin Elmer) and Chromatography Interface 600 series (Perkin Elmer) comprise the instrumentation used for HPLC studies. The temperature of the column was maintained at 25°C using a thermostatted column case (Timberline Instruments, Inc, Boulder, CO).

All stock solutions (standard Hepes/NaCl buffer, 6M GuHCl and 8M urea) were first filtered using a 0.45µm nylon filter membrane (Sigma Aldrich, MO) and degassed in helium for 5 mins at 10psi prior to use to prevent the introduction of air bubbles in to the column. The column was first flushed with buffer for at least 60 mins prior to the addition of denaturant (four column volumes). For unfolding and refolding studies, the column was then equilibrated at each denaturant concentration for at least 30 mins (two column volumes) prior to sample injection. The denatured and renatured protein samples used were prepared according to Section 2.2.3. Samples (20µl) of 2.5×10^{-7} M BPAO in each of the varying denaturant solvents were individually injected into the sample port. Elution of the protein samples was monitored spectrophotometrically at 250 nm.

Elution volumes (V_e) for the observed protein peaks were calculated from their measured retention times (R_t) and column flow rate (R_f):

$$V_e = (R_t) * (R_f) \quad (34)$$

The flow rate, chosen for the resolution of monomers and dimers of BPAO, was determined from the specific resolution (R_{sp}) of two standard proteins. The standard proteins selected were aldolase (158 kD) and ovalbumin (45 kD) as their molecular weights are close to that of the native BPAO dimer (192kD) and the expected monomeric species (86kD), respectively. The specific resolution (R_{sp}) of these two proteins on the column was calculated by the equation [249]:

$$R_{sp} = \frac{2(V_{e_2} - V_{e_1})}{(V_{p_2} + V_{p_1}) \log \left(\frac{M_1}{M_2} \right)} \quad (63)$$

where: V_{e_1} and V_{e_2} are the elution volumes of known MW proteins 1 and 2; V_{p_1} and V_{p_2} are the peak volumes of proteins 1 and 2; and M_1 and M_2 are their respective molecular weights. For the BioSep S3000 column, the highest R_{sp} value possible was 2.23 at 0.30 ml/min. Therefore, all experiments were performed at this flow rate.

The void (V_0) and total volumes (V_t) of the column were determined from the elution volumes for blue dextran (2000kD) and β -mercaptoethanol (78.13 g/mol), respectively, in the absence of protein. The performance of the column was unaffected by the addition of 6M GuHCl or 8M urea with no change to the measured void ($V_0 = 1.98 \pm 0.03$ ml) and total volumes ($V_t = 4.18 \pm 0.05$ ml).

The pressure in the column was 360 ± 14 psi in buffer, 463 ± 29 psi in 8M urea and 523 ± 29 psi in 5M GuHCl, which was much lower than that used for hydrostatic pressure measurements of denaturation of proteins and therefore assumed to have no significant effect on BPAO. The varying column pressures may result from differences in the

viscosities of the solvent. Kawahara *et al* [248] have shown that the relative viscosity of 5M GuHCl to water is 1.408 (Table 9) and for 8M urea it is 1.663.

2.2.6.2 Data Analysis

From the measured void (V_0) and total column (V_t) volumes and V_e for the protein elution, the distribution constant (K_{av}) for the protein is given by:

$$K_{av} = \frac{(V_e - V_o)}{(V_t - V_o)} \quad (64)$$

Stokes radii (R_s) and molecular weights (M) were determined by linear evaluations of experimental K_{av} and R_s or M . The Stokes radii (R_s), determined from standards (Table 10), have an r^2 of 0.9595 and were calculated by:

$$\log R_s = -1.317K_{av} + 2.147 \quad (65)$$

The molecular weight values (M) were determined from the standards (Table 10). Standard molecular weights were fit to the equation ($r^2 = 0.8967$):

$$\text{Log}M = -2.855K_{av} + 6.158 \quad (66)$$

Table 10
Standards used in HPLC experiments

Standards	Stokes Radius (Rs, Å)	Molecular Weight (M, kD)
Ribonuclease	19.3	13.7
Chymotrypsinogen A	21.7	25
Ovalbumin	30.2	43.5
Bovine Serum Albumin (BSA)	33.9	67
Aldolase	46.6	158

Adapted from Uversky VN (1993) Use of fast protein size-exclusion liquid chromatography to study the unfolding of proteins which denature through the molten globule. *Biochemistry* 32(48): 13288-13298 [250].

2.2.7 Polyacrylamide gel electrophoresis (PAGE)

2.2.7.1 Sample Preparation

Native PAGE: Slab gels were prepared using a 4% (w/v) acrylamide stacking gel with 0.125M Tris-HCl, pH 6.8 [251]. The separating gel (dimensions 8 x 8 x 0.075 cm) was prepared with a gradient of 5 – 10% (w/v) acrylamide in 0.375M Tris-HCl, pH 8.8. The ratio of acrylamide to bisacrylamide was kept at a constant 1:30. Polymerization was initiated using ammonium persulfate (APS) and N, N, N', N'-tetramethylethylenediamine (TEMED). For the gradient gel, 0.16% (w/v) sucrose was added to the solution with the highest concentration of acrylamide (10% w/v). This stabilized the gradient which was mixed and pumped using an LKB Bromma 2120 Varioperpex[®] II Pump.

Native BPAO samples were prepared in 0.03M Tris-HCl pH 6.8 with 10% (w/v) glycerol used to increase the density of the sample. For native gel electrophoresis, the molecular weight protein markers (Amersham Pharmacia Biotech Inc, NJ) used were: thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD) and bovine serum albumin (67 kD).

SDS-PAGE: Slab gels were prepared using a 4% (w/v) stacking gel with 0.125M Tris-HCl and 0.05% (1.73mM) SDS at pH 6.8 [251]. The separating gels were composed of a gradient of 8 – 18% (w/v) acrylamide in 0.375M Tris-HCl and 0.05% SDS at pH 8.8. The ratio of acrylamide to bisacrylamide was maintained at a constant mole ratio of 1:30. Gel polymerization was achieved using 0.05% (w/v) APS and 0.05% (v/v) TEMED. Approximately 0.16% (w/v) sucrose was added to the 18% (w/v) acrylamide solution to stabilize the gradient.

Protein samples for SDS-PAGE analysis were prepared in 0.0625M Tris-HCl, 0.05% (1.73mM) SDS, pH 6.8 with 10% glycerol. In some cases, the protein samples were heated to 95°C for 5 mins prior to loading on the gel. Low molecular weight reference proteins (Amersham Pharmacia Biotech Inc, NJ) used for SDS-PAGE were phosphorylase b (97 kD), bovine serum albumin (BSA) (66 kD), ovalbumin (45 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20 kD) and α -lactalbumin (14.4 kD). Additionally, a wider range of protein molecular weight standards were used for some analyses (Novex, CA) and included: myosin (200 kD); b-galactosidase (116.3 kD); phosphorylase b (97.4 kD); bovine serum albumin (66.4 kD); glutamic dehydrogenase (55.4 kD); lactate dehydrogenase (36.5 kD); carbonic anhydrase (31kD); trypsin inhibitor (21.5kD); lysozyme (14.4kD); aprotinin (6kD); insulin B chain (3.5kD); and insulin A chain (2.5kD).

Transverse urea gradient gel electrophoresis (TUGGE): Urea gels were prepared according to the method of Creighton [216] with slight modifications. Polyacrylamide slab gels (8 x 8 x 0.15 cm) were prepared with a transverse 0 – 8M urea gradient (Figure 17). A peristaltic pump and two-chamber gradient maker were used to form the gradient of the heavier 8M urea solution into the glass plates before 0M urea. The acrylamide concentration in the 0M urea solution was 11% (w/v) and in the 8M urea was 7% (w/v) to compensate for the reduced mobility in higher urea concentrations due to greater viscosity [215]. The buffer used was 0.375M Tris-HCl, pH 8.8 [217]. The gels were polymerized with 10% ammonium persulfate and TEMED and could be stored at 4°C for one day without significant dissipation of the urea gradient [215].

2.2.7.2 Electrophoretic conditions

Electrophoresis was performed using a Hoeffer SE 260 Mighty Small II for about 2 hours in the electrophoresis running buffer (0.025M Tris and 0.192M glycine, pH 8.3). For SDS electrophoresis, the running buffer contained also 0.05% SDS (w/v). Cooling water was used to maintain the temperature of the buffer chamber. During migration of the bromophenol blue tracking dye through the stacking gel, the current was maintained at 15mA. Afterwards, the current was increased to 20mA as the tracking dye migrated through the 0.75 mm thick separating gel.

For TUGGE, protein samples (50 μ g) were incubated in 0.0583M Tris-HCl, pH 6.8 with 5% glycerol for 5 mins prior to loading onto the gel. Electrophoresis was performed on a Hoeffer SE 260 Mighty Small II (Pharmacia Biotech, CA) for about 3 hours in 0.025M Tris and 0.192M glycine, pH 8.3. Cooling was achieved with circulating cold water. The current was kept at a constant 10mA until the bromophenol blue tracking dye migrated about 5mm into the transverse gel. Afterwards, the current was increased to 30mA for the 1.5mm thick gel.

2.2.7.3 Staining of gels

Gels were stained for protein bands using either the “Rapid Coomassie Blue” or “Coomassie Brilliant Blue” staining procedures [252]. Both techniques use Coomassie Brilliant Blue G 250. As the name suggests, the first method can be accomplished within 2 hours but there was some loss of sensitivity. Throughout both procedures, the gel was agitated slowly on a rocking platform. With the rapid Coomassie blue method, the gel

was fixed using an isopropanol fixing solution for 30 - 60 mins, stained with rapid Coomassie blue solution (0.006% w/v) for 2 or more hours and then destained with 10% (v/v) acetic acid.

For the alternate Coomassie blue staining method, the gel was first fixed in a methanol solution for 2 hours, followed by staining with Coomassie blue solution (0.025% w/v) for 4 hours. Gels were then destained for 2 or more hours until a clear background with blue bands was obtained. All gels were scanned using a Hewlett Packard ScanJet 6100C scanner to maintain a permanent record and then stored in deionized water at 4°C.

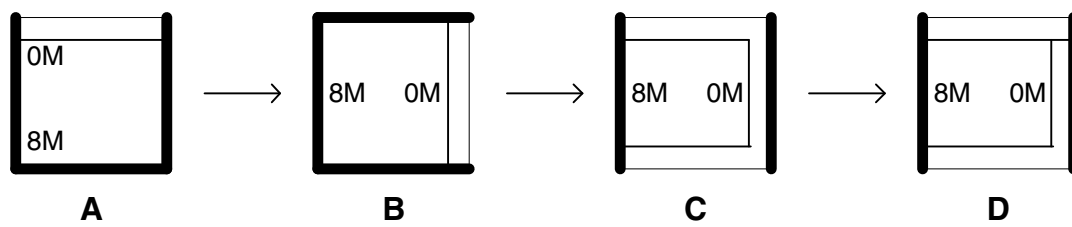


Figure 17: Overview of urea gel preparation. A: assembly of the three 1.5mm spacers on the sides and bottom of the glass plates and pouring of the gel. B: 90° rotation of the gel. C: removal of the top and bottom spacers, followed by insertion of a side spacer. D: sealing the additional area of the gel with 0M urea solution.

2.2.8 Data Analysis

For studies of protein folding and dissociation, retrieved parameters (y) from steady state (CSM, integrated intensity, $\langle r \rangle_{ss}$ and total intensity), time resolved studies (β , ϕ , $\langle r \rangle_{TR}$ and τ), specific activity (SA_0/SA) and HPLC (V_e and R_s) data were plotted as a function of denaturant concentration (x). Where appropriate, the parameters were fit (Sigma Plot 7.0) to a two-state model (dimer-to-monomer or dimer-to-dimer unfolded) using the following general sigmoidal expression:

$$y = y_0 + \frac{a}{\left[1 + e^{-\left(\frac{x-x_0}{b} \right)} \right]} \quad (67)$$

where a and y_0 are the asymptotic maximum and minimum values respectively, b is the slope parameter and x_0 is the denaturant concentration at the inflection point. Similarly, all hyperbolic transitions were fit to the equation:

$$y = y_0 + \frac{ax}{b+x} \quad (68)$$

with y_0 and a as the abscissa minimum and maximum, respectively, and b is the ordinate value at 50% change in y . The fraction of native protein (f_N) was calculated by the equation:

$$f_N = \frac{y_D - y}{y_N - y_D} \quad (69)$$

where y_N and y_D are the experimental values of the native and denatured states, respectively.

The correlation coefficient (r^2) was used to determine the quality of the fit. For the experiments reported here, an r^2 value greater than 0.98, unless otherwise noted, was judged an appropriate fit.

2.2.9 Thermodynamic Analysis of Protein Unfolding

The free energy for the transition from a dimer to dissociated state or an unfolded dimeric conformation can be calculated using either the linear extrapolation method or a non-linear fit.

Model 1: Dimer Dissociation $N_2 \rightarrow 2M$

For the dissociation of a dimeric protein into monomers, the fraction of denatured protein (f_U) was determined from Equation 70, where y is the observed experimental data (CSM, $\langle r \rangle$, residual activity, R_S , normalized Integrated Intensity) and y_N and y_D are the experimental values for the native and denatured states, respectively. The equilibrium constant for dissociation, K_D , was calculated by Equation 7 using the total monomer concentration, P_t . Free energies (ΔG) were then calculated from the K_D values at each denaturant concentration by Equation 8 using the gas constant (R) and the absolute temperature, T . Values of ΔG in the transition region were then used to determine the free energy in the absence of denaturant, $\Delta G_{H_2O}^0$, by Equation 4, using the linear extrapolation method. Alternatively Equation 12 was fit to the entire denaturant range to resolve $\Delta G_{H_2O}^0$ and m [68]. The transition midpoint for dissociation (C_m) was then calculated from Equation 9.

$$f_U = \frac{y_N - y}{y_N - y_D} \quad (70)$$

$$K_D = 2P_t \left[\frac{f_U^2}{1 - f_U} \right] \quad (7)$$

$$\Delta G = -RT \ln K_D \quad (8)$$

$$\Delta G = \Delta G_{H_2O}^0 - m[D] \quad (4)$$

$$y = y_N - (y_N - y_D) \frac{\left(\sqrt{\frac{1 + 8P_T}{e^{-\left(\frac{\Delta G_{H_2O}^0 - m[D]}{RT}\right)}}} - 1 \right)}{4P_T} e^{-\left(\frac{\Delta G_{H_2O}^0 - m[D]}{RT}\right)} \quad (12)$$

$$C_m = \frac{RT \ln[P_t] + \Delta G_{H_2O}^0}{m} \quad (9)$$

Model 2: Dimeric Unfolding $N_2 \rightarrow I_2$

For a dimeric protein that undergoes unfolding rather than dissociation, the linear extrapolation method as well as a non-linear fit can be used to describe the data. The unfolding constant, K_U , is determined by Equation 71 and is used to calculate the free energy (ΔG) at each denaturant concentration (Equation 3) in the transition region by the linear extrapolation method. The free energy in the absence of denaturant ($\Delta G_{H_2O}^0$) is determined by Equation 4 and the denaturant concentration at the transition midpoint for unfolding is calculated by Equation 5.

$$K_U = f_U = \frac{y_N - y}{y_N - y_D} \quad (71)$$

$$\Delta G = -RT \ln K_U \quad (3)$$

$$\Delta G = \Delta G_{H_2O}^0 - m[D] \quad (4)$$

$$C_m = \frac{\Delta G_{H_2O}^0}{m} \quad (5)$$

Alternatively, denaturation by unfolding using a direct non-linear fit can usually be fit to two different states, the native (N) to the denatured state (D) (N→D). In addition, thermodynamic data, specifically the free energy of denaturation, $\Delta G_{H_2O}^0$, is analyzed from equilibrium denaturation curves fit to a two-state [64], a two-state transition with pre- and post-transition regions [65] or three-state transitions [253]. In the simplest model, a two-state transition, experimental data, y , is fit to Equation 10 [64], where y_N and y_D are the intercepts for the native and denatured states, $\Delta G_{H_2O}^0$ is the free energy for the denaturation transition in the absence of denaturant, m is the slope of the denaturation transition, $[D]$ is the denaturant concentration, R is the gas constant (1.987 cal.M⁻¹.K⁻¹ or 8.314 J.M⁻¹.K⁻¹) and T is the absolute temperature.

$$y = \frac{y_N + y_D e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}}}{1 + e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}}} \quad (10)$$

The best fit of the four parameters (y_N , y_D , $\Delta G_{H_2O}^0$, m) was determined by non-linear regression using Sigma Plot 7.0. Alternatively, another two-state transition (Equation 11) includes additional parameters, m_N and m_U for the slopes of the pre- and post-denaturation data [65]. The six parameters (y_N , y_D , $\Delta G_{H_2O}^0$, m , m_N , m_D) were determined using Sigma Plot 7.0.

$$y = \frac{\left[(y_N + m_N[D]) + (y_D + m_D[D]) e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}} \right]}{1 + e^{\frac{-(\Delta G_{H_2O}^0 + m[D])}{RT}}} \quad (11)$$

The total free energy for a dimer is calculated by:

$$\Delta G_{total} = \sum \Delta G_U^D + \Delta G_D + 2\Delta G_U^M \quad (72)$$

where ΔG_U^D and ΔG_U^M are the free energy of unfolding for the dimer and monomer, respectively and ΔG_D is the free energy of dissociation of the dimer. Similarly, the total m value (m_{total}) is the sum of the m values for dimer unfolding (m_U^D), dissociation (m_D) and monomeric unfolding (m_U^M) by the equation:

$$m_{total} = \sum m_U^D + m_D + 2m_U^M \quad (73)$$

The goodness of fit is determined by the correlation coefficient, r^2 , where >0.99 was considered an excellent fit.

Chapter 3: Results and Discussion

3.1 Characterization of BPAO Purity

The purity and homogeneity of BPAO after protein purification was monitored by PAGE, HPLC and enzyme kinetic analyses (Methods Sections 2.2.7, 2.2.6 and 2.2.1). The surface character of the protein was accessed from ANS and bis-ANS binding studies (Methods Section 1.5.1.1.2.2).

3.1.1 Electrophoresis: Native polyacrylamide gel electrophoresis (Native-PAGE) revealed a protein band (Figure 18) corresponding to a molecular weight value of around 140 kD (lane 2). Based on the molecular weight of the amino acid sequence, the anticipated molecular weight is 170kD. The difference may arise due to the non-spherical conformation of BPAO [113].

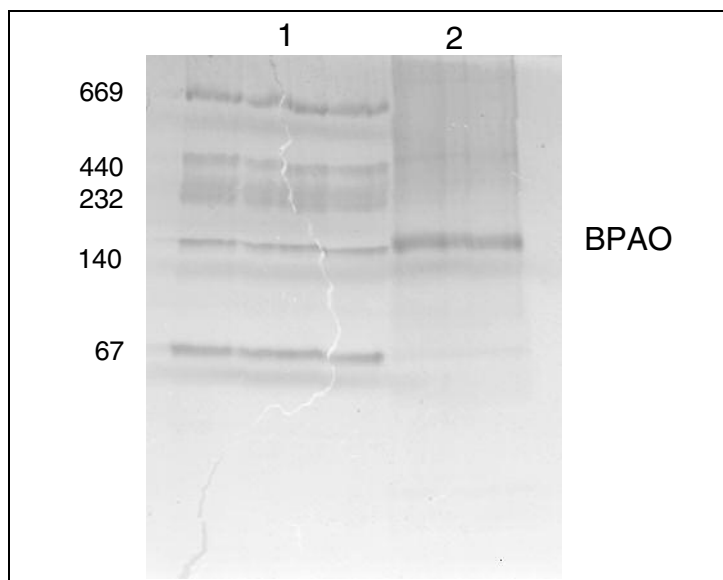


Figure 18: Native-PAGE of purified BPAO. The 0.75mm thick gel has 5-10% acrylamide. Lane 1 represents the reference proteins with molecular weights shown to the left and lane 2 is 20μg BPAO. The gel was stained using the rapid Coomassie blue technique.

3.1.2 HPLC: The HPLC elution chromatogram for native BPAO suggested greater than 90% protein purity (Figure 49). A major peak eluting at 8.81mins ($V_e = 2.64\text{ml}$), corresponding to the purified dimeric BPAO protein, was clearly observed. The molecular weight for the protein peak was equivalent to 192kD (Equation 66). This value is consistent with previous literature reports [146, 160]. A minor peak was routinely observed to elute after 6.40mins ($V_e = 1.92\text{ml}$) and from molecular weight analyses, was presumed to represent aggregated protein species.

3.1.3 Enzyme Kinetics: The Michaelis-Menten constant (K_m) is a constant for a particular protein and substrate (S) under defined conditions (temperature, pH) and can provide an estimation of enzyme (E) purity by comparison with literature values.

A plot of the velocity (v) of BPAO *versus* benzylamine concentration ($[S]$) is shown in Figure 19. As the concentration of benzylamine was increased, the expected hyperbolic increase in the specific activity of BPAO was observed. From data fitting to the Michaelis-Menten expression (Equation 39), a value of 9.4mM for K_m was retrieved for BPAO with benzylamine at 25°C and pH 7.5.

Alternatively, analysis of the data using the double reciprocal Lineweaver-Burk plot (Equation 40) of the Michaelis-Menten equation for the substrate range of $0.33K_m$ to $2K_m$ provided a linear fit (shown as the *inset*, Figure 19), with a high correlation coefficient ($r^2 = 0.9825$). Parameters retrieved from the fit were: $V_{\max} = 0.00554\text{s}^{-1}$ and $K_m = 11.4\text{mM}$.

K_m values for the BPAO purified in this study are five-fold higher than for those reported in published studies (Table 11). Given that the PAGE analysis suggests that

BPAO has been purified to apparent homogeneity (Figure 18), the higher retrieved K_m values obtained here may arise from the presence of low concentrations of non-enzymatically active protein aggregates, as suggested from HPLC analyses (Figure 49). Alternatively, it is possible that very low concentrations of aniline hydrochloride, used to elute BPAO off the ω -aminohexyl agarose affinity column, remained in the protein solution even after desalting for at least 3 times (Section 2.2.2). Aniline hydrochloride is an efficient competitive inhibitor of BPAO with an inhibition constant, K_i , of 4.3mM [146].

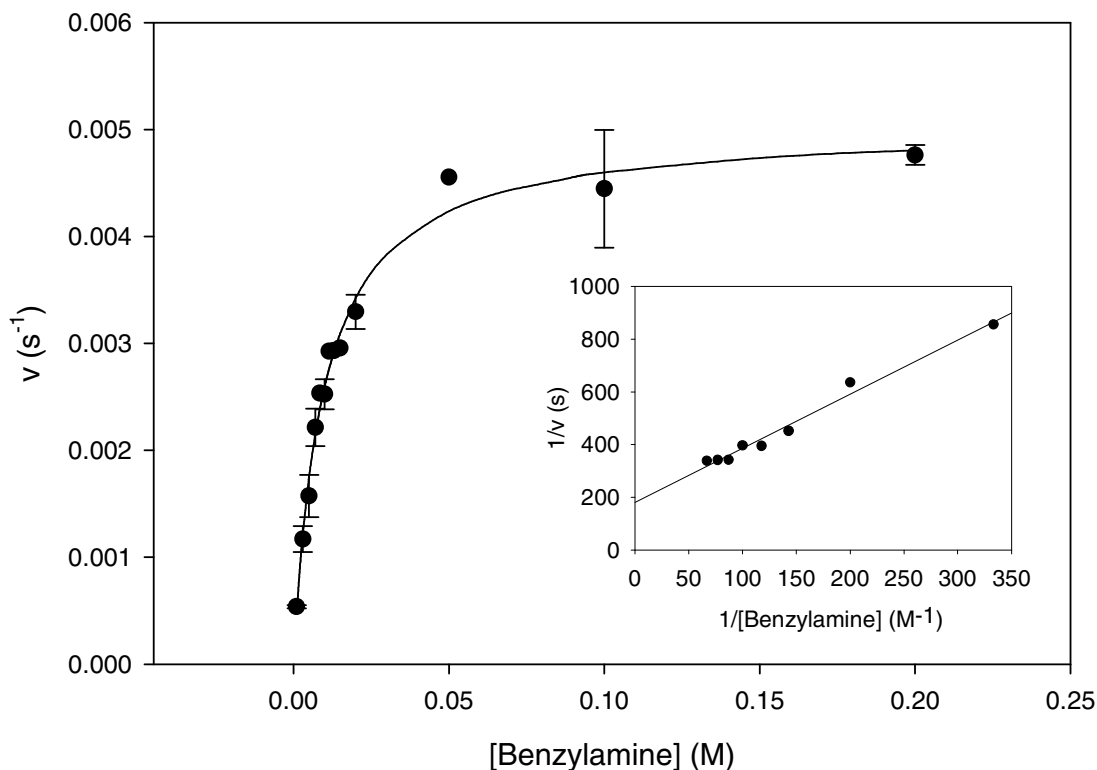


Figure 19: The effect of benzylamine concentration on the specific activity of native BPAO in 0.02M Hepes with 0.1M NaCl buffer, pH 7.5 at 25°C. The BPAO concentration was fixed at $2.5 \times 10^{-7}\text{M}$. The line is a fit using Michaelis-Menton kinetics (Equation 39) to the data with $r^2 = 0.9872$ and K_m and $V_{\max}$ of 0.0094M benzylamine and 0.0050s^{-1} , respectively. *Inset*: The corresponding Lineweaver-Burk double reciprocal plot. The line represents a linear fit of the data with a correlation coefficient, r^2 , of 0.9825. The recovered $V_{\max}$ and K_m values are 0.00554s^{-1} and 0.0114M benzylamine, respectively, from Equation 40.

Table 11:
Summary of reported and experimentally obtained Michaelis-Menten constants (K_m)
for BPAO with benzylamine.

K_m (mM)	Conditions	References
^a 9.4/11.4	0.02M Hepes with 0.1M NaCl pH 7.5, 25°C	This study
2.2	0.1M potassium phosphate buffer pH 7.2, 25°C	[158]
1.9±0.1	0.1M potassium phosphate buffer pH 7.2, 25°C	[111]
1.9	0.1M potassium phosphate buffer pH 7.2, 25°C	[119]
1.7	0.1M potassium phosphate buffer pH 7.2, 25°C	[119]
1.68	0.1M potassium phosphate buffer pH 7.2, 25°C	[254]
1.5	0.025M Hepes/0.15M NaCl pH 7.2, 37°C	[144]
0.74±0.05	30mM sodium phosphate buffer pH 7.4	[255]
0.73	100mM phosphate buffer pH 7.2, 25°C	[256]

^aValues for K_m for BPAO were obtained from data fitting to the Michaelis-Menten (Equation 39) and the Lineweaver-Burke (Equation 40) equations, respectively.

3.1.4 Surface Character of BPAO (ANS and Bis-ANS Binding):

The binding of the hydrophobic fluorophores, ANS and bis-ANS, to BPAO was examined to determine the hydrophobic character of the native protein. Figure 20 shows the titration of bis-ANS with BPAO. An increase in BPAO concentration results in an increase in the fluorescence emission intensity from bis-ANS on binding to the protein. The fluorescence intensity of bis-ANS and ANS in solution is negligible. A value for $\Delta F_{\max}$ (Section 2.2.5.1.1) was obtained from the double reciprocal plot of fluorescence intensity (observed at 500nm for bis-ANS) *versus* protein concentration (Figures 21). The $\Delta F_{\max}$ value corresponds to 1.53×10^6 .

The binding of bis-ANS and ANS to BPAO is shown in Figures 22 and 24. Each figure reflects a hyperbolic saturation nature of the fluorescence intensity at 500nm and 471nm for bis-ANS or ANS binding, respectively. Further analyses of the data using Equation 54 and 55 provided the dissociation constant (K_D) (Figures 23 and 25). For bis-ANS, K_D values of $10.2 \times 10^{-6} \text{M}$ (Equation 54) and $7.9 \times 10^{-6} \text{M}$ (Equation 55) were calculated. ANS (Figure 25) shows comparable binding characteristics compared to bis-ANS (Figure 23), with slightly higher K_D values of $1.26 \pm 0.14 \times 10^{-5} \text{M}$ (Equation 54) and $1.33 \pm 0.27 \times 10^{-5} \text{M}$ (Equation 55).

It is interesting to note that although bis-ANS is a dimer of ANS (Figure 15B) and thus twice as large, bis-ANS binding to BPAO is comparable with that of ANS. The K_D for pig plasma amine oxidase (PPAO) with ANS ($5 \mu\text{M}$) [233] is reported to be ~ 2.5 times lower than that of BPAO ($1.33 \pm 0.27 \times 10^{-5} \text{M}$ (Equation 55, Figure 25)). This may result from differences in protein structure and hydrophobic character.

BPAO has exposed hydrophobic patches on the native protein surface to which the hydrophobic probes, ANS and bis-ANS, can bind with varying affinities. A summary of the number of ANS and bis-ANS bound to proteins and the corresponding K_D values are shown in Table 7. Hydrophobic proteins, which exhibit tight binding with ANS (BSA [186] and bacteriorhodopsin [21]) and bis-ANS (α -crystallin [22]), have K_D values of $\sim 1\mu\text{M}$. This is about 10-fold lower than the K_D values of ANS or bis-ANS binding to BPAO.

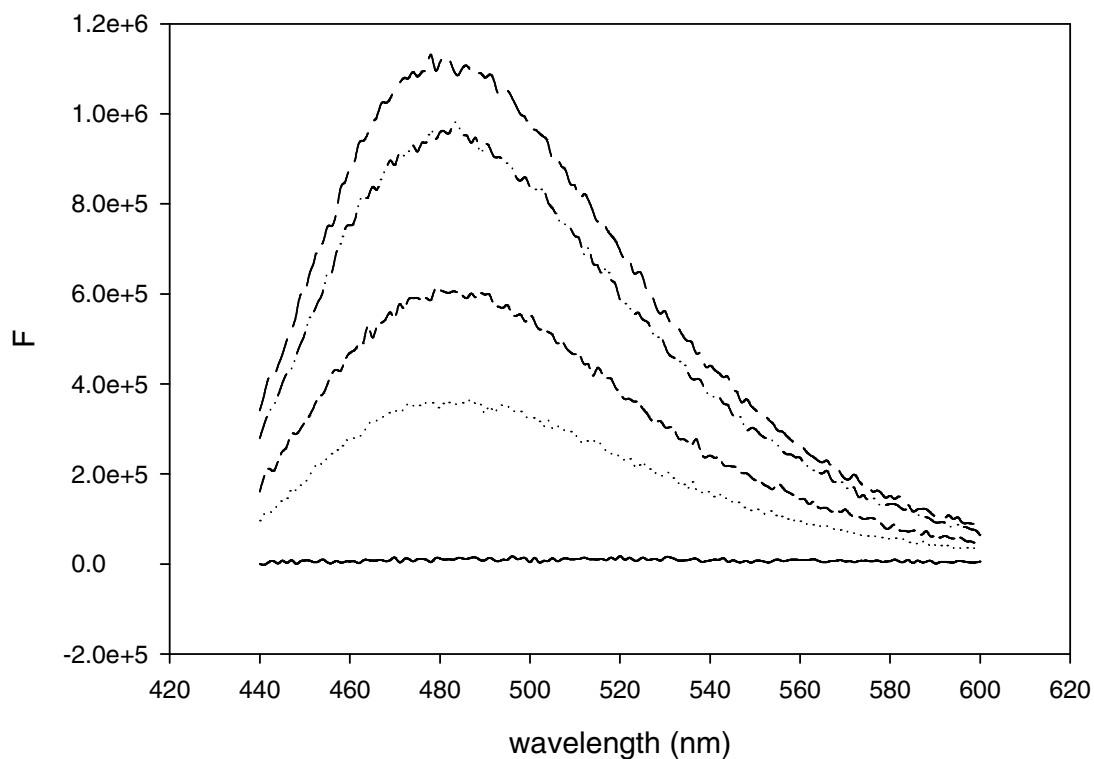


Figure 20: The effect of titrating increasing BPAO concentration into bis-ANS. The concentration of bis-ANS is $3.3 \times 10^{-7} \text{ M}$ and the concentrations of BPAO from bottom to top are 0, 0.159, 0.32, 0.653 and 1.01 mM. The samples are excited at 395 nm. The change in volume with titration is less than 5%. Excitation and emission path lengths are 10 mm and 2 mm, respectively.

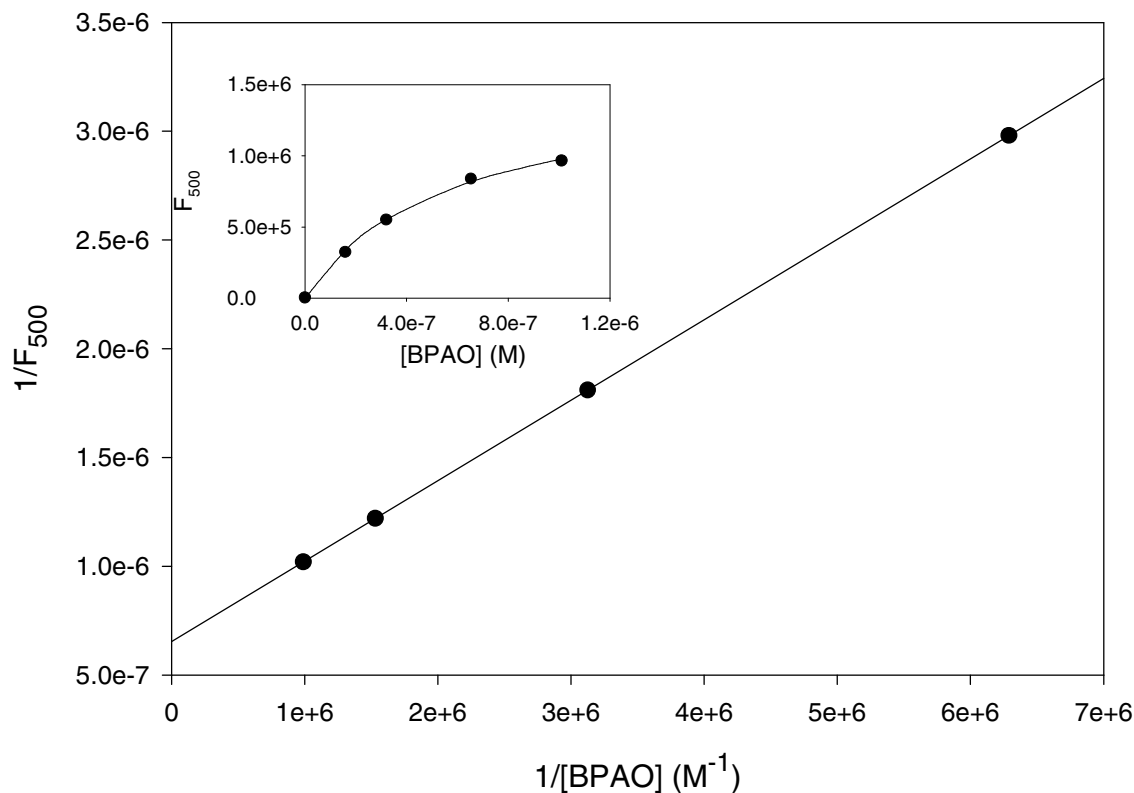


Figure 21: Double reciprocal plot of fluorescence emission intensity of bis-ANS and BPAO concentration. The concentration of bis-ANS is $3.3 \times 10^{-7} M$. The samples are excited at 395nm and emission is monitored at 500nm. Excitation and emission path lengths are 10mm and 2mm, respectively. The $\Delta F_{\max}$ is 1.53×10^6 with excitation at 10mm. *Inset*: The effect of BPAO concentration on the fluorescence emission intensity of bis-ANS. The line shows the hyperbolic fit to the data (Equation 68).

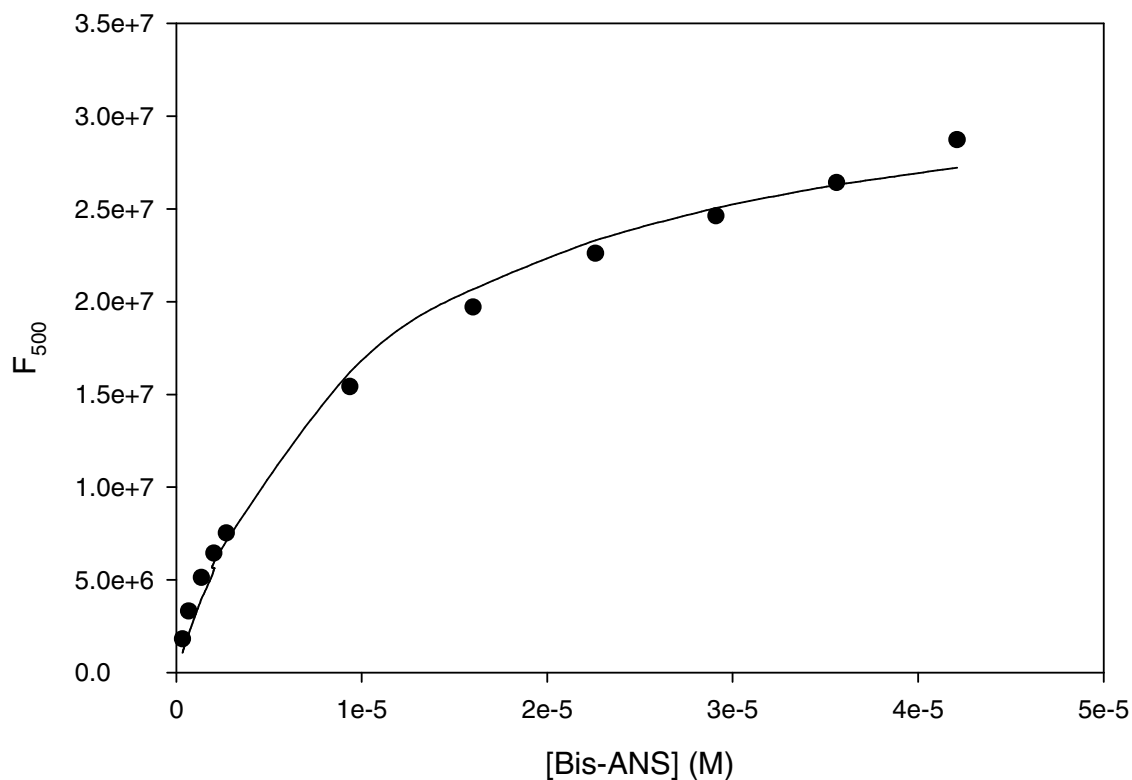


Figure 22: Titration of BPAO by bis-ANS. The concentration of BPAO is $1.01 \times 10^{-6}\text{M}$. The line represents the hyperbolic fit (Equation 68) with a ΔF_{max} of 3.38×10^7 and K_D of $1.02 \times 10^{-5}\text{M}$. Bis-ANS is excited at 395nm and the data is corrected for the contribution from free bis-ANS. Excitation and emission path lengths were 2mm and 10mm, respectively. The maximum molar ratio of bis-ANS to BPAO is 42.

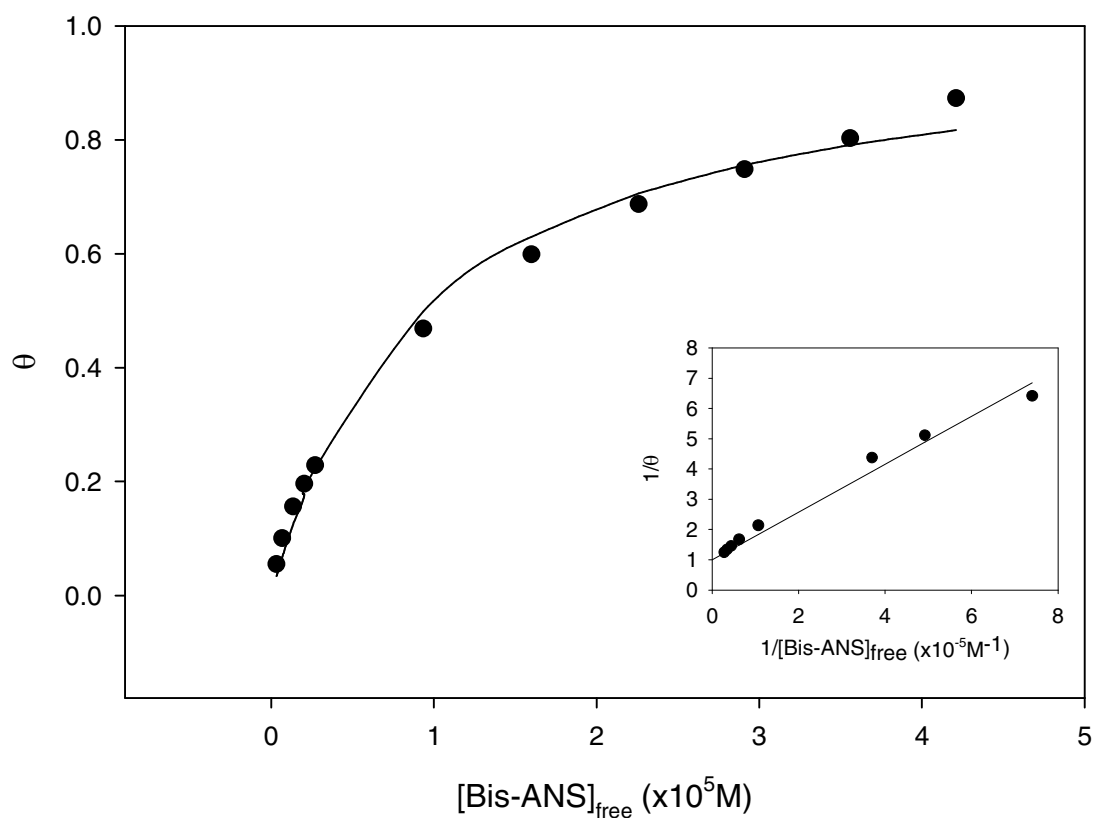


Figure 23: Analysis of bis-ANS binding to BPAO. The $[\text{BPAO}]$ is $1.01 \times 10^{-6} \text{M}$ and θ is calculated by Equation 46. The line represents the fit to Equation 54 ($r^2 = 0.9911$) with a K_D ($1/K_A$) of $1.02 \times 10^{-5} \text{M}$. *Inset:* The double reciprocal plot. The line is the fit to the data to Equation 55 ($r^2 = 0.9819$). The K_D is $7.9 \times 10^{-6} \text{M}$.

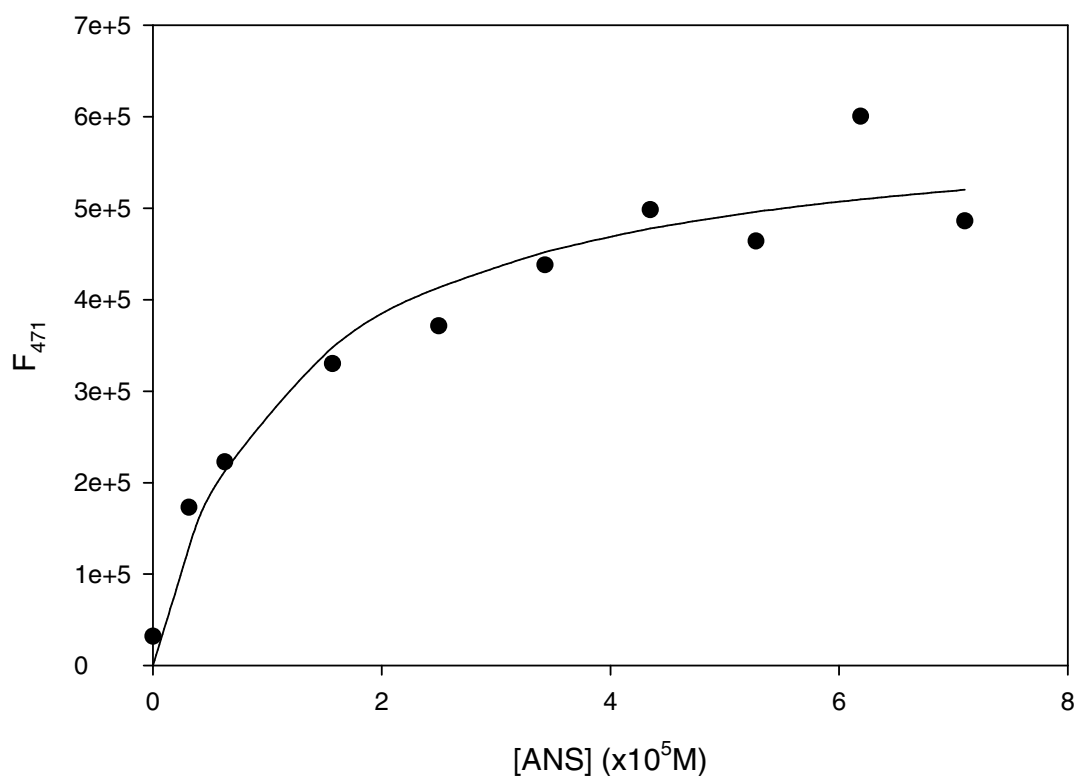


Figure 24: Effect of ANS concentration on BPAO fluorescence emission intensity at 471nm. BPAO concentration is $2.80 \times 10^{-7} \text{M}$. ANS is excited at 370nm with excitation and emission path lengths of 2 and 10mm, respectively. BPAO fluorescence intensity is corrected for the contribution from free ANS. The line represents the hyperbolic trend (Equation 68) with ΔF_{max} at 6.1×10^5 and a 50% change in F_{471} at $1.2 \times 10^{-5} \text{M}$ ANS. The maximum molar ratio of ANS to BPAO is 254.

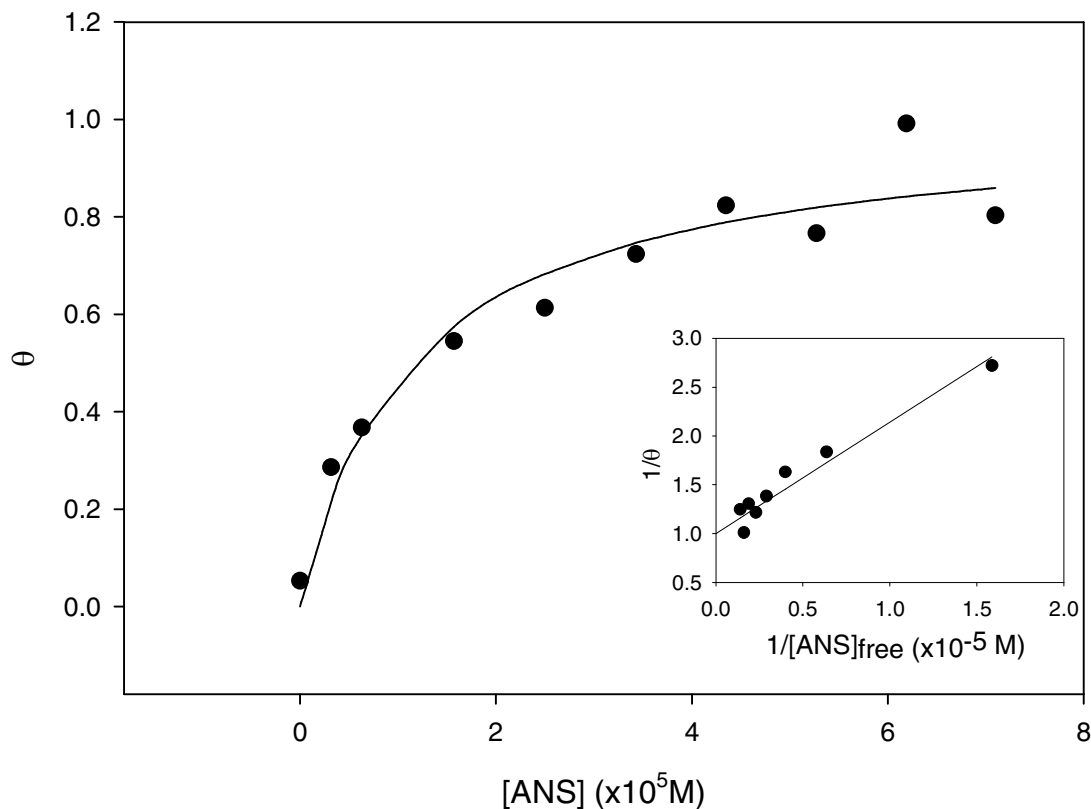


Figure 25: Analysis of ANS binding to BPAO. The $[BPAO]$ is $2.80 \times 10^{-7} \text{ M}$, θ is calculated by Equation 46 and ΔF_{max} was determined from Figure 24. The line represents the fit to Equation 54 ($r^2 = 0.9407$). The K_D ($1/K_A$) calculated from 2 trials is $1.26 \pm 0.14 \times 10^{-5} \text{ M}$. *Inset:* The double reciprocal plot. The line is the fit to the data to Equation 55 ($r^2 = 0.9506$). The K_D is $1.33 \pm 0.27 \times 10^{-5} \text{ M}$ from 2 trials.

3.2 Conformational Studies of BPAO

The dissociation and unfolding pathway for BPAO was explored using gel electrophoresis [215], fluorescence spectroscopy [257] and liquid chromatography [258] methodologies. For a large multidomain dimeric protein different techniques provide sensitivity to different aspects of the unfolding/dissociation pathway and conformational intermediates.

3.2.1 Gel Electrophoresis

3.2.1.1 Urea Gradient Gel Electrophoresis (UGGE): The dissociation of multimeric proteins may be visualized from UGGE and has been used previously to detect the presence of monomeric [87, 217] or dimeric [218] species in response to incubation with differing urea concentrations. For BPAO, the electrophoretic pattern with a urea gradient from 3 to 8M is shown in Figure 26. There is an aggregate band (A) at the top of the gel. At urea concentrations 0M to 4M, a single protein band was observed (N) and represents the native dimeric conformation for BPAO. Interestingly, for the urea concentration range 4M – 5.5M, a second slower moving BPAO band (U) is observed in equilibrium with the N band which may not be attributed to protein aggregates (A). Since the observed transition from native (N) dimeric to unfolded (U) BPAO on the UGGE gel is not a continuous two-state transition, the data suggests that the unfolding is incomplete within the experimental time (~3hours). For a dimer (N) to monomer dissociation, a band of increased mobility would be expected for higher urea concentrations corresponding to the lower molecular weight species. However, for BPAO the electrophoretic mobility of the resultant second protein band (U) is decreased relative to the dimer (N) on migration

at higher urea concentrations. Such an effect may represent higher multimers of BPAO, unfolding of the BPAO dimer to an alternative dimeric conformational state or an unfolded monomer with a large hydrodynamic size. Further experiments were performed to distinguish between the three possibilities. At urea concentrations greater than 5.5M, the BPAO conformation represented by the U band predominates the electrophoretic pattern.

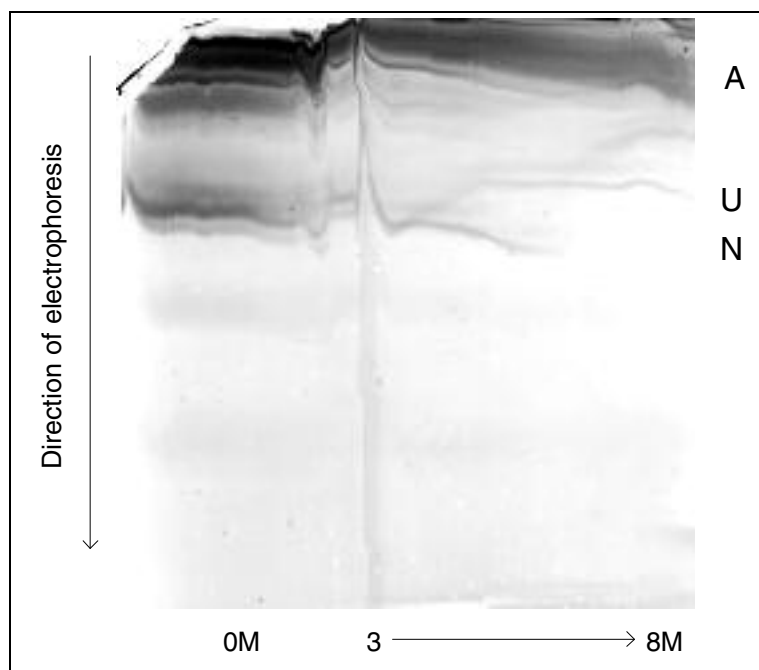


Figure 26: Transverse urea gradient gel electrophoresis of BPAO. Protein concentration was 3mg.mL^{-1} or $1.5 \times 10^{-5}\text{M}$. A 11-7% (w/v) acrylamide gradient was superimposed on the 3-8M urea gradient. The gel is 1.5mm thick. N represents the native dimeric BPAO conformation while U is the urea denatured conformation and A represents aggregates. The gel was stained using the rapid Coomassie blue technique.

3.2.1.2 SDS-PAGE Analysis of BPAO: The influence of SDS on the dissociation of BPAO was visualized from SDS-PAGE (Figure 27). Addition of 0.05% (w/v) SDS (1.73 mM) to BPAO resulted in the appearance of a single predominant protein band on SDS-PAGE corresponding to a molecular weight of 97 kD (conformation A) suggesting dissociation of homodimeric BPAO (Molecular weight = 192 kD from HPLC (Figure 49)) to the monomer. Dissociation was achieved at room temperature without the addition of reducing agents (lane 3). Heating the protein solution at 100°C for 5 minutes prior to loading on the gel results in a slight decrease in the intensity of impurity bands (B, C and D) but no change in the A conformation of the BPAO monomer (lane 2). Of most interest is the presence of the low concentration A' band found only in the BPAO sample incubated at room temperature. This A' band is not due to the presence of dimers, but more likely is an alternate monomeric conformation which is in equilibrium with conformation A, the main monomeric band. Apparently, incubation at high temperatures is necessary to shift the equilibrium towards conformation A. Based on the intensity of the A and A' bands, the concentration of A' is very low compared with that of A.

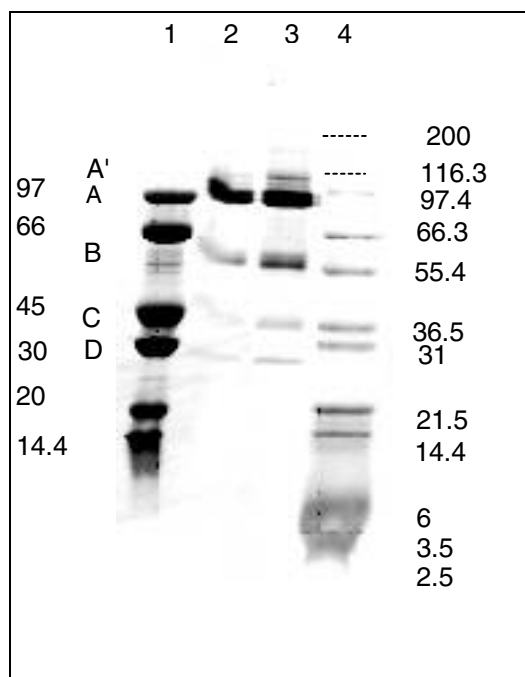


Figure 27: Non-reducing 0.05% (w/v) or 1.73mM SDS-PAGE of BPAO. Lanes 2 and 3 show monomeric BPAO in non-reducing conditions at 100°C and 25°C, respectively. Molecular weight protein references are shown in lanes 1 and 4 with the corresponding molecular weights on the left and right, respectively. The letters (A', A, B, C and D) reflect the different BPAO bands. For clarity, the dotted lines show the references at 116.3 and 200 kD which were too faint to be seen from the scan. The acrylamide gradient is 8 – 18% (w/v), the gel is 0.75mm thick and 20µg BPAO is loaded per lane. Staining was accomplished with the coomassie brilliant blue method.

3.2.2 Analysis of Protein Conformation Using Fluorescence Methods

Intrinsic and extrinsic fluorophores are used to monitor global and local motions in proteins. The fluorescence intensity of a fluorophore is an important parameter that can provide valuable information about the global structure of a protein. Furthermore, emission anisotropy studies yield information about the molecular dimension of proteins.

3.2.2.1 Tryptophan

3.2.2.1.1 Intensity Studies: Dimeric BPAO has 30 tryptophan residues, which can each serve as a fluorescent reporter of their local protein environment. As a result, the measured fluorescence signal for BPAO represents a weighted average arising from each tryptophan residue and reflects the contributions from the many fluorophore microenvironments within the protein. The change in the averaged tryptophan fluorescence intensity and emission spectrum with altered protein conformation can provide insights into unfolding and dissociation pathways.

Figure 28 shows the different effects that GuHCl, urea and SDS have on BPAO conformation as detected from changes in the tryptophan emission spectrum of BPAO. Native BPAO in Hepes/NaCl buffer shows a fluorescence intensity maximum around 340 nm (λ_{max}). This represents a folded protein conformation [Section 1.5.1.1.1]. Incubation of the protein with 8M urea ($\lambda_{\text{max}} = 355$ nm) or 6M GuHCl ($\lambda_{\text{max}} = 355$ nm) results in a significant (15 nm) red-shift in the fluorescence emission spectra arising from the exposure of buried tryptophan residues to solvent during denaturation of the protein [71, 75, 79, 82, 163, 166]. In contrast, addition of SDS to BPAO shows a slight blue shift ($\lambda_{\text{max}} = 334$ nm) in the fluorescence emission maximum with respect to the native protein.

This may arise from the interaction of the hydrophobic SDS tail with the protein [33, 34], essentially shielding the tryptophan residues of the protein from solvent.

Analyses of conformational intermediates in the dissociation and/or unfolding pathways of BPAO from tryptophan fluorescence emission spectra were achieved by quantifying the observed wavelength shifts and changes in fluorescence intensity as the center of spectral mass parameter (CSM, Equation 17). The effects of denaturants (GuHCl, urea and SDS) on the tryptophan emission CSM parameter are shown in Figures 29, 30 and 31, respectively.

In the case of GuHCl (Figure 29) and urea (Figure 30), the spectral data clearly shows a two state transition. Two pathways may be proposed for interpretation of the data. The first model involves a transition from the BPAO dimer to the monomer and the second, as a dimer to an unfolded-dimeric conformation. In an attempt to resolve these two pathways, the effect of varying the BPAO concentration on the transition midpoint was investigated. From the Law of Mass Action (Equation 7), a concentration dependence of the transition would suggest a dimer-monomer two-state transition.

For GuHCl (Figure 29), the BPAO concentration was increased twelve-fold from 0.213 μM to 2.5 μM . With increasing concentrations of GuHCl (0 – 5M), the sigmoidal increase in the tryptophan CSM value from the native conformation (346.6 ± 1.1 nm) to an unfolded form (358nm) were superimposable for both protein concentrations examined, with transition midpoints (C_m) at 2.10 ± 0.02 and 2.15M GuHCl, respectively. Since the unfolding process is independent of protein concentration, this suggests that BPAO perturbation by GuHCl follows the uni-molecular unfolding process rather than aggregation or dissociation. However, it is possible that a 12-fold change in protein

concentration was not sufficient to shift the observed transition, (particularly if the dissociation constant is small) and a broader range of protein concentrations may be necessary.

A similar result was observed for the urea studies (Figure 30). Only slight changes (within experimental error) in the denaturation transition midpoint was observed (5.12 to $5.66 \pm 0.21\text{M}$) when the BPAO concentration was increased 20-fold. This again suggests that BPAO prefers to undergo dimeric unfolding rather than dissociation in the presence of urea. However, like GuHCl, it is possible that a 20-fold change in protein concentration was not sufficient to affect the observed transition.

Refolding of BPAO from GuHCl and urea was also investigated from analysis of shifts in the tryptophan fluorescence emission spectra (Figure 29 and 30). BPAO was renatured by dilution to the desired denaturant concentration from GuHCl (5M) or urea (8M) with constant unfolded protein concentration in each sample (See Methods 2.2.3). Both GuHCl and urea treated protein samples underwent refolding. However, hysteresis was observed in the sigmoidal CSM refolding data with a shift in the mid-transition denaturant concentration to lower values compared to the corresponding unfolding direction (Figure 29 and 30). This suggests that the refolding of BPAO to an alternate stable state is by a different pathway.

From SDS studies, the CSM parameter represents only a small emission spectral shift for the tryptophan residues of BPAO, from ~ 346 nm for the native protein to 349 nm in 14mM SDS (Figure 31), in contrast to a CSM value of $\sim 358\text{nm}$ in 5M GuHCl and 8M urea (Figures 29 and 30). The small changes in CSM values with addition of SDS occur around the cmc concentration for SDS in the Hepes buffer used here ($\sim 1\text{mM}$) [29].

From the SDS-PAGE analysis using 1.73mM SDS (Section 3.2.1.2), we noted that BPAO exists exclusively in the monomeric form at the concentrations used here. However, while the protein has undergone dissociation, the resultant monomers appear to exist in a folded conformation with shielding of the tryptophan residues from solvent.

In addition to monitoring changes in the CSM values for BPAO, the effect of altered protein conformation was also investigated from effects on tryptophan intensity, which is related to the averaged fluorescence lifetime (Equation 19). Intensities were derived from integration of the fluorescence emission spectra, and then normalized to those values obtained for the highest denaturant concentration. The overall sensitivity in the normalized integrated fluorescence emission intensities for denaturation and renaturation of BPAO, in GuHCl and urea, was less than recorded for CSM values (*insets* in Figures 29 and 30, respectively). Neither GuHCl nor urea showed any significant intensity effects with unfolding and refolding. BPAO can be refolded, but the final conformation represents an alternate but stable conformation for the dimeric protein.

For BPAO with SDS, the normalized integrated intensities (Figure 32) revealed a two-phase sigmoidal quenching effect (30%) with a transition midpoint at 3.13mM, which is above the cmc of ~1mM [29]. Previous SDS-PAGE studies (Section 3.2.1.2) suggest that BPAO exists exclusively in the monomeric form at these SDS concentrations and that the observed intensity transition may reflect unfolding of BPAO monomers. Such a clear intensity transition was not observed for urea and GuHCl.

The decrease in SDS integrated intensity suggests the quenching of tryptophan residues by SDS molecules. Analysis of the intensity data using a Stern-Volmer plot (Equation 18) was non-linear (data not shown). A modified Stern-Volmer plot (Equation

21, Figure 33) was used to determine the fraction of initial fluorescence accessible to SDS interactions (0.82) and the quenching constant (0.029M^{-1}).

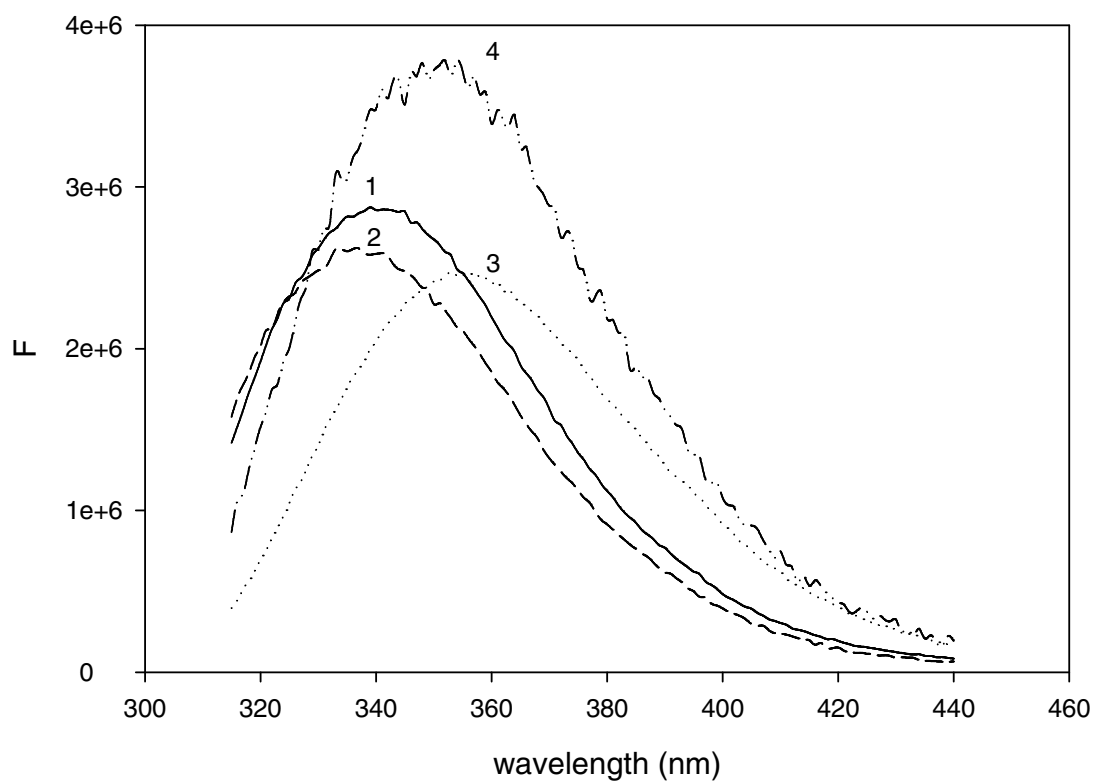


Figure 28: Fluorescence emission spectra of tryptophan in BPAO in various denaturants. The concentration of BPAO is 2.5×10^{-7} M and the solvents are: (1) Hepes/NaCl buffer; (2) 0.014M SDS; (3) 6M GuHCl; and (4) 8M urea. The tryptophan residues were excited at 295nm and the slits used for excitation and emission were 10nm and 2nm, respectively.

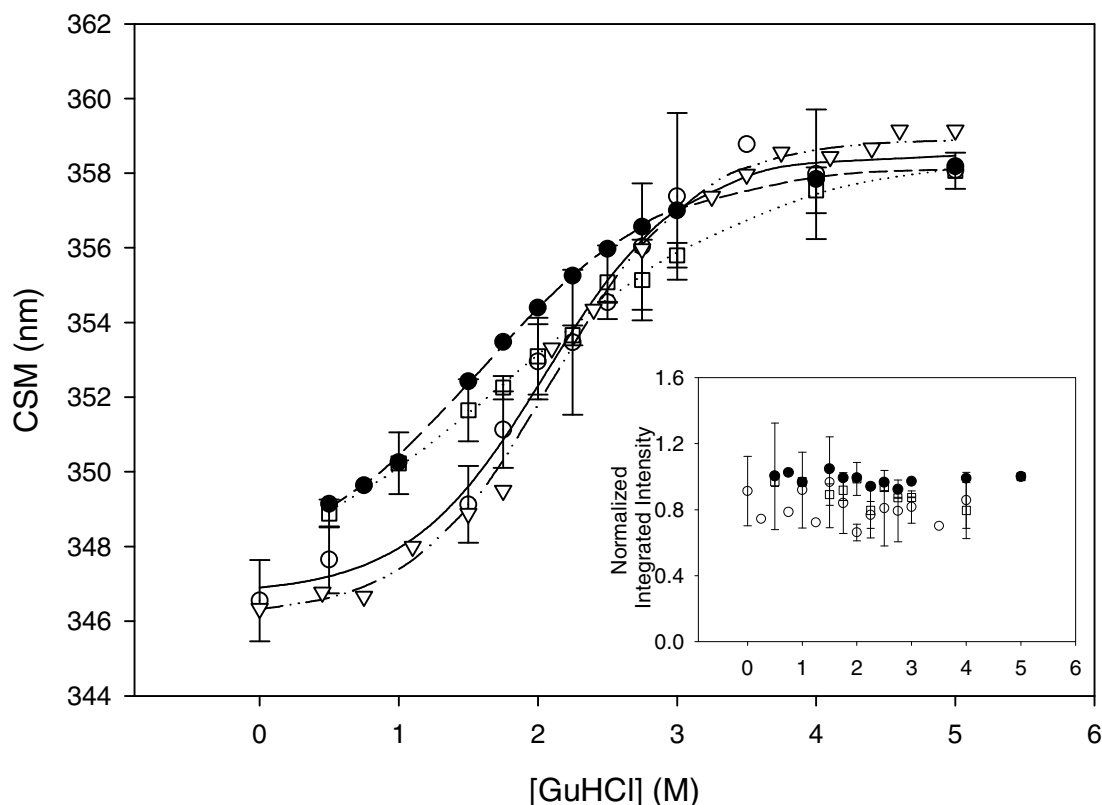


Figure 29: Effect of GuHCl on the tryptophan fluorescence emission spectra of denatured and renatured BPAO. The protein concentration for the denatured samples was $2.13 \pm 0.53 \times 10^{-7}$ ($\circ$) and 2.5×10^{-6} M (∇) while for renatured samples it was $1.69 \pm 0.27 \times 10^{-7}$ ($\square$) and 1.87×10^{-6} M ($\bullet$). Both renatured ($\square$) and denatured ($\circ$) data shows the standard deviations from 2 trials each. The lines represent a sigmoidal fit to the data (Equation 67) with transition midpoints at 2.10 ± 0.02 M ($\circ$), 2.11 M (∇), 1.80 ± 0.09 M ($\square$) and 1.59 M ($\bullet$) GuHCl. BPAO samples were excited at 295 nm and the CSM was calculated from the emission range 315–440 nm (Equation 17). *Inset:* The effect of GuHCl on the corresponding integrated intensity of the tryptophan emission spectra. Integrated intensity is normalized at 5 M GuHCl and is calculated by Equation 53. The 2.5×10^{-6} M BPAO CSM data is courtesy of Reuven Yakubov.

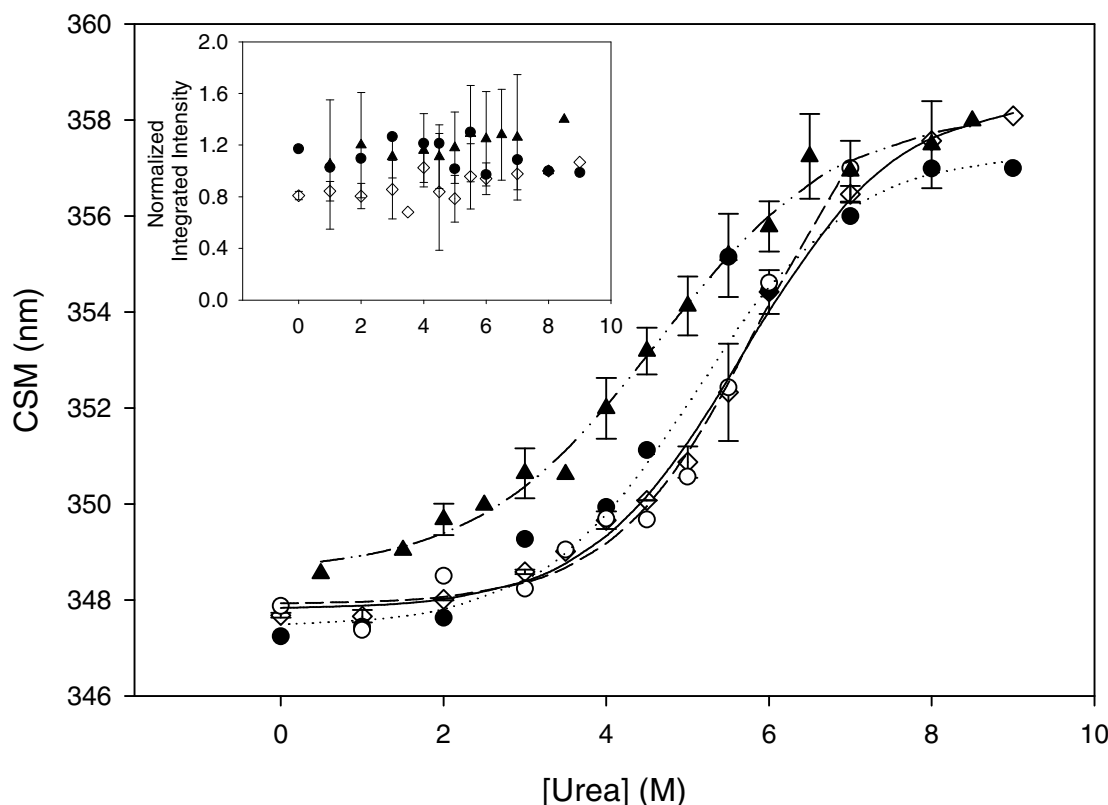


Figure 30: Effect of urea on the tryptophan fluorescence emission spectra of denatured and renatured BPAO. The renatured protein concentration is $2.68 \pm 0.96 \times 10^{-7} \text{ M}$ (▲) and denatured concentrations are $5.00 \times 10^{-8} \text{ M}$ (●), $1.00 \times 10^{-7} \text{ M}$ (○) and $1.00 \times 10^{-6} \text{ M}$ (◇). Renatured (▲) and denatured (◇) BPAO shows the standard deviations from 3 and 2 trials respectively. The lines represent a sigmoidal fit (Equation 67) to the data with transition midpoints at $4.79 \pm 0.07 \text{ M}$ (▲), 5.12 M (●), 5.93 M (○), and $5.66 \pm 0.21 \text{ M}$ (◇) urea. BPAO samples were excited at 295nm and the CSM was calculated from the emission range 315-440nm (Equation 17). *Inset:* The effect of urea on the corresponding integrated intensity of the tryptophan emission spectra. The integrated intensity is normalized at 8M urea and calculated by Equation 53.

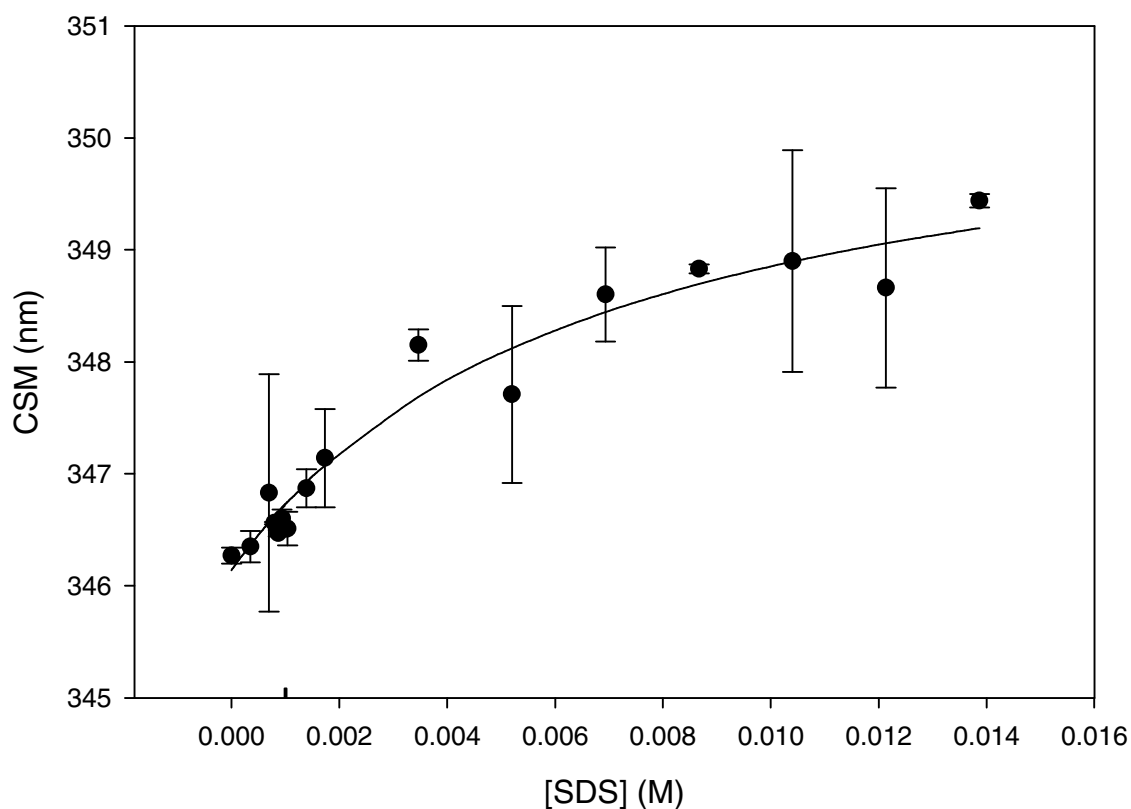


Figure 31: Effect of SDS on the tryptophan emission spectra of denatured BPAO. The concentration of BPAO is $4.57 \pm 1.13 \times 10^{-7}$ M. BPAO samples were excited at 295nm and the CSM was calculated from the emission range 315-440nm (Equation 17). For clarity, the line shows a hyperbolic fit (Equation 68) to the data with a 50% change in CSM at 6.67×10^{-3} M SDS for 5 trials. The cmc at 0.12M ionic strength is shown by the vertical line at 1mM SDS [29].

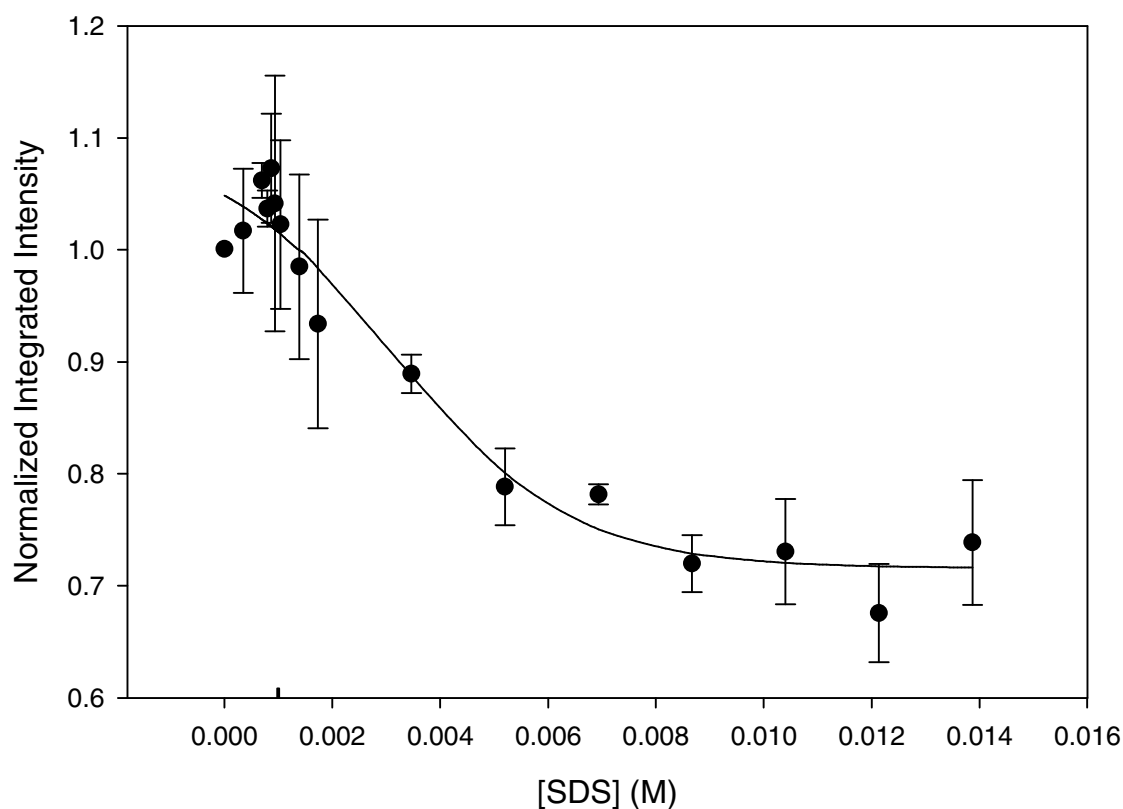


Figure 32: The effect of SDS on the integrated fluorescence intensity of the BPAO emission spectra. Integrated intensity was normalized at 0M SDS and calculated by Equation 53. The protein concentration was $4.57 \pm 1.13 \times 10^{-7} \text{M}$. The line shows the sigmoidal fit ($r^2 = 0.9553$; Equation 67) to the data with a transition midpoint at $3.13 \times 10^{-3} \text{M}$ SDS. The standard deviations reflect 5 trials. The cmc at 0.12M ionic strength is shown by the vertical line at 1mM SDS [29].

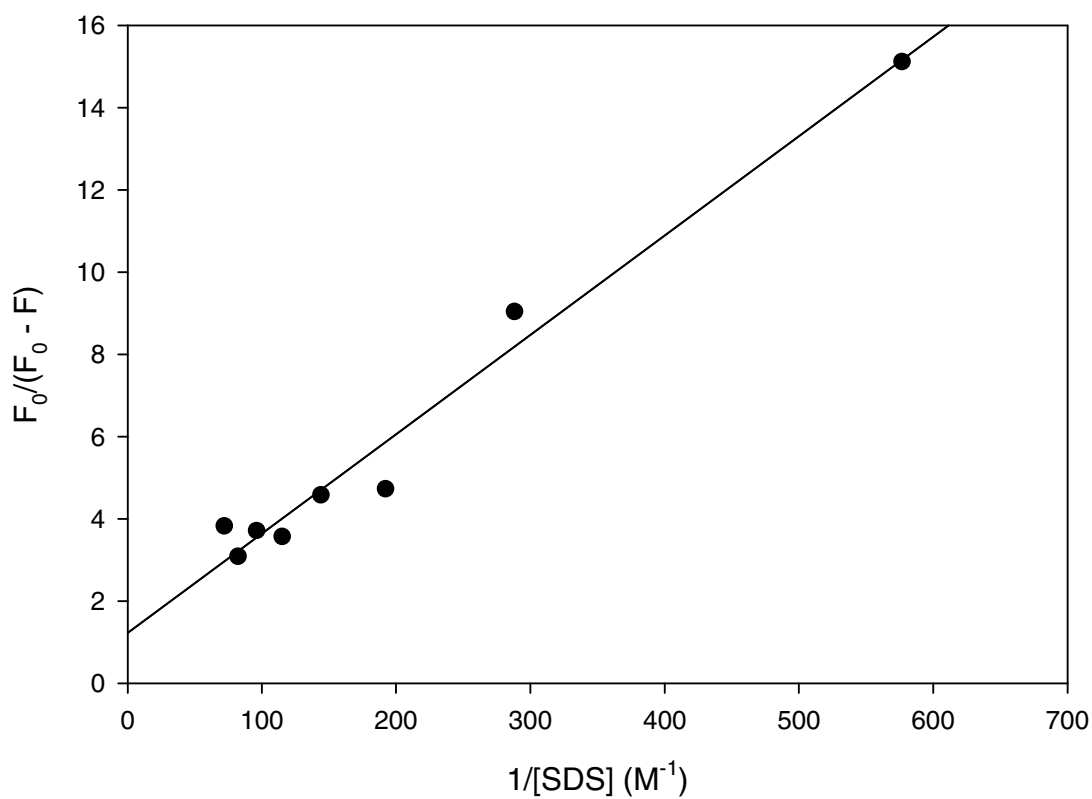


Figure 33: Modified Stern-Volmer plot for tryptophan fluorescence quenching of BPAO by SDS. The fraction of tryptophan accessible (f_a) is 0.816 and the quenching constant (K_Q) is 0.0294M^{-1} , as determined by Equation 21. The correlation coefficient is 0.9749.

3.2.2.2.1 Steady-state Fluorescence Emission Anisotropy: Fluorescence emission anisotropy values provide information about the rotational volume of a protein (Equation 25). This is an important parameter when determining the molecular weight of a protein (Equation 26), and consequently the unfolding or dissociation pathway of a protein. Using the Perrin equation (Equation 25), it is possible to estimate the expected rotational correlation time ($\phi = 64\text{ns}$) for a dimeric protein with a molecular weight of 192kD, and hence 32ns for the monomer (96 kD), assuming a spherical protein with a specific volume (v) of 0.73 g/ml. However, the average fluorescence lifetime (τ) for tryptophan residues in proteins is on the order of 5ns (e.g. Barstar [171]) and thus the fluorescence time window is expected to provide sensitivity to rotational motions occurring in a time of less than $\sim 15\text{ ns}$ (3τ). Since the fluorescence lifetime of tryptophan is significantly less than the predicted rotational correlation times for BPAO ($\tau \ll \phi$), the steady state fluorescence emission anisotropy values for tryptophan residues of BPAO are not expected to resolve rotations of the dimeric and monomeric forms of the protein. Rather, the anisotropy may exhibit sensitivity to more rapid rotational motions, such as local unfolding of protein domains. BPAO has three domains per monomer (Figure 5), so the anisotropy of tryptophans in BPAO has the potential to monitor local flexibility by analysis of the average population of tryptophan residues.

The effect of GuHCl on the emission anisotropy of tryptophan residues in BPAO is shown in Figure 34. For native dimeric BPAO, the anisotropy value measured for the tryptophans was 0.166. The value corresponds to a species with a molecular weight estimated to be 73kD (Equations 25 and 26), assuming a limiting anisotropy value (r_0) for tryptophan of 0.2 [171] with an average fluorescence lifetime, τ , of 5ns [171]. This large

underestimation of the true molecular weight confirms the insensitivity of tryptophan anisotropy to changes in the global structure of BPAO.

As further denaturant is added, a sigmoidal decrease in anisotropy values was observed (Figure 34) with a midpoint for the transition (C_m) at 2.42M GuHCl, which is a slightly higher value (by 0.5M) than recorded from CSM data (Figure 29). At 5M GuHCl, the measured emission anisotropy for the denatured BPAO is 0.080, which suggests a more rotationally unrestricted environment for the tryptophan residues of the protein. This final anisotropy value has not been corrected for any lifetime changes (see *Inset*, Figure 34) and the true value is most likely lower than reported here.

The renaturation of the protein was also followed from emission anisotropy studies of tryptophan (Figure 34). Refolding also revealed a sigmoidal change (Equation 67) with a transition mid-point concentration of 1.44M GuHCl, which is lower than recovered for the unfolding direction (2.42M). The hysteresis in the refolding curve leads to a decrease in the tryptophan anisotropy of refolded compared to unfolded BPAO for an equivalent GuHCl concentration. While it is clear from anisotropy data that BPAO may be refolded from GuHCl, it is also important to note that this refolding pathway is different from the unfolding direction. This is further supported by the corresponding total fluorescence intensity (Equation 24) data (*inset*, Figure 34). For denaturation, a sigmoidal decrease in intensity was observed (C_m at 1.67M GuHCl), whereas during refolding the normalized total fluorescence intensity remained constant as the concentration of GuHCl is decreased from 5M to 0.5M. This suggests different conformational states for BPAO during unfolding and refolding.

Unfolding experiments with increasing urea concentrations (Figure 35) also showed a sigmoidal decrease (Equation 67) in emission anisotropy values for BPAO from 0.154 to 0.070 with a transition midpoint occurring at 4.03M urea. Interestingly, the normalized total fluorescence intensity values (Figure 35, *inset*) revealed an increase upon protein unfolding. This fluorescence increase suggests a relief of quenching by the local amino acid environment surrounding the tryptophan residues upon unfolding. It would appear that the unfolding pathways for BPAO may be quite different in response to the addition of urea or GuHCl.

For anionic SDS, though no specific trends in the tryptophan emission anisotropy were observed for BPAO, there was a decrease in fluorescence intensity (Figure 36 and *inset*, respectively). The small decrease in emission anisotropy values, ranging from 0.164 to a value of 0.130 at 12mM SDS suggests an overall greater rotational restriction of the tryptophan residues of BPAO in SDS. This is in contrast to tryptophan emission anisotropy values observed for BPAO in 5M GuHCl and 8M urea ($\langle r \rangle = 0.082$ and 0.070, respectively; Figures 34 and 35). Our previous SDS-PAGE results (Figure 27) suggest that BPAO exists as monomers at SDS concentrations greater than 1.73 mM. Clearly, as discussed above, the relatively short fluorescence lifetime of tryptophan compromises the sensitivity of the anisotropy to the dimer-monomer dissociation. Nevertheless, altered tryptophan emission anisotropy values observed with GuHCl and urea most likely reflects local unfolding effects.

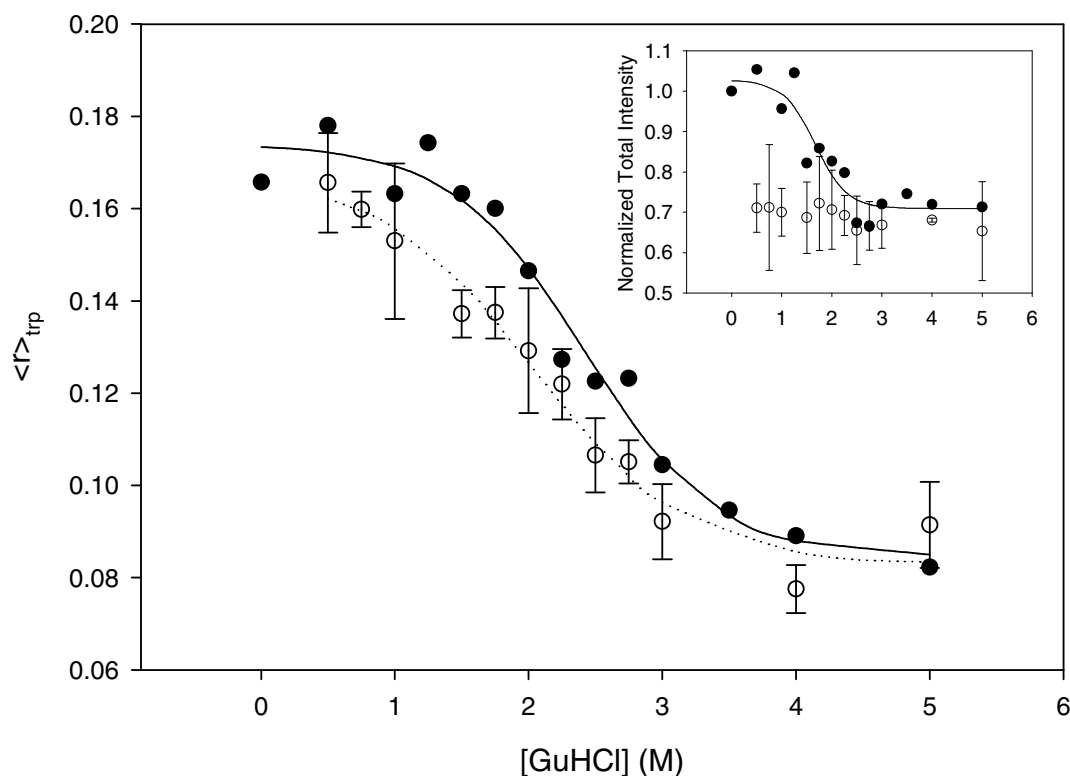


Figure 34: The effect of GuHCl concentration on tryptophan emission anisotropy. The concentration of BPAO in denatured (●) and renatured (○) solutions is $2.5 \times 10^{-7}\text{M}$. Anisotropy is collected at 350nm with excitation at 295nm and calculated by Equation 22. The lines represent the sigmoidal fit (Equation 67) to the data with transition midpoints at 2.42 (●) and 1.44M (○) GuHCl. *Inset:* The effect of GuHCl concentration on tryptophan total fluorescence intensity (Equation 24). The data is normalized at 0M GuHCl. The line represents a sigmoidal fit (Equation 67) for denaturation with a transition midpoint of 1.67M GuHCl.

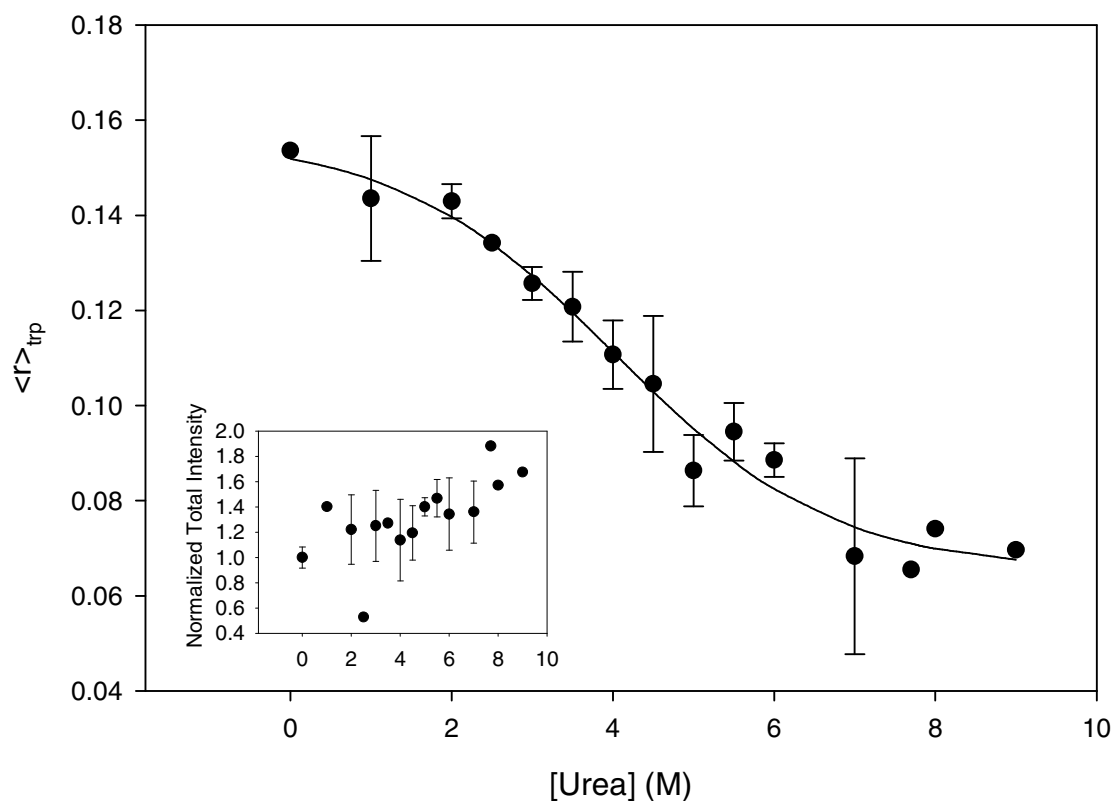


Figure 35: The effect of urea concentration on tryptophan emission anisotropy. The concentration of BPAO in the denatured solution is $2.5 \times 10^{-7}\text{M}$ (●). Anisotropy is collected at 350nm with excitation at 295nm and calculated by Equation 22. The line represents a sigmoidal fit to the data with transition midpoint at 4.03M urea. *Inset*: The normalized total fluorescence intensity is calculated by Equation 24 and normalized at 0M urea.

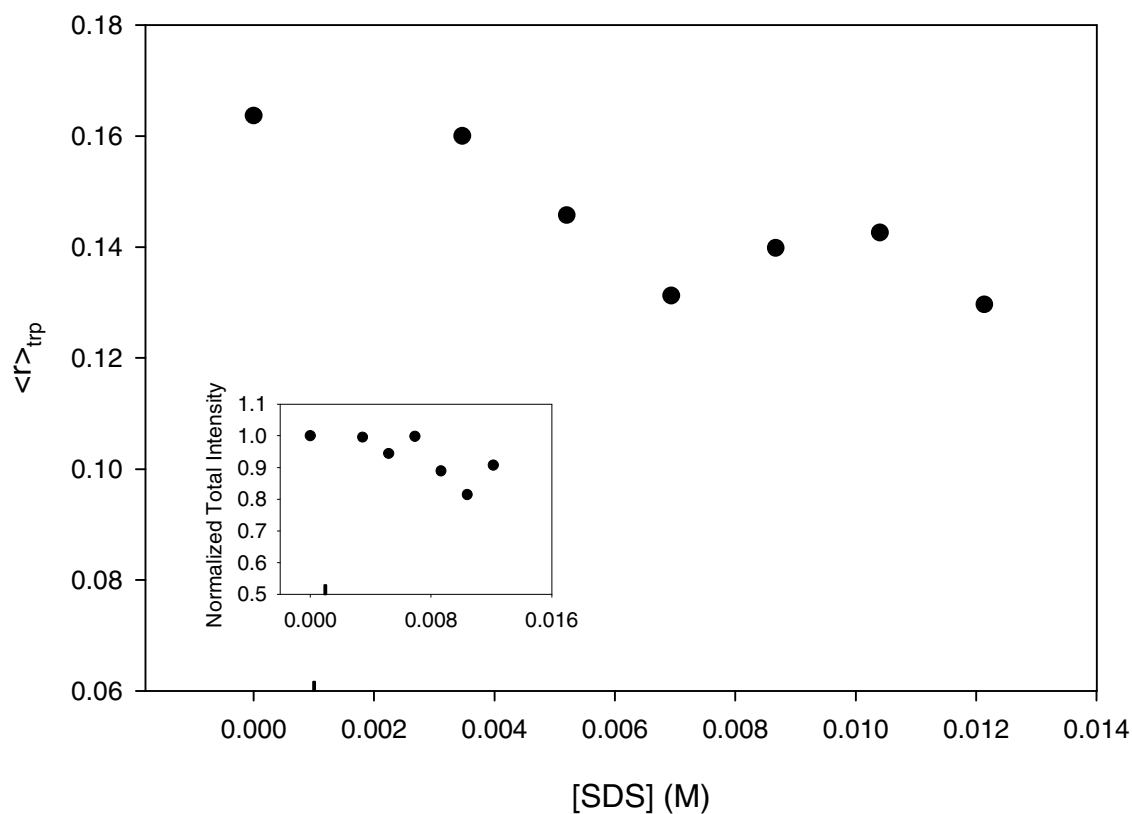


Figure 36: The effect of SDS concentration on tryptophan emission anisotropy. The concentration of BPAO in the denatured solution is $2.5 \times 10^{-7} \text{M}$ (●). Emission anisotropy is collected at 350nm with excitation at 295nm and calculated by Equation 22. *Inset*: The normalized total fluorescence intensity is calculated from Equation 24 and normalized at 0M SDS. The cmc at 0.12M ionic strength is shown by the vertical line at 1mM SDS [29].

3.2.2.2 Pyrene maleimide

3.2.2.2.1 Steady State Fluorescence Emission Spectra

In addition to using the tryptophan residues of BPAO as intrinsic reporter groups for both local and global conformation of the protein, extrinsic probes can also be utilized. One such probe is pyrene maleimide (PM) [173], which selectively binds to free –SH groups in proteins. In addition, pyrene has a long average fluorescence lifetime (>30 ns) [181-183] that makes it an attractive fluorophore for conformational/dissociation studies of high molecular weight proteins. Unlike studies using tryptophan anisotropy, the experimental time window is considerably extended for pyrene and hence allows studies of the slower rotational motions of larger macromolecular assemblies.

From spectroscopic analyses (Section 2.2.4) pyrene maleimide was conjugated with free cysteine residues (C94 from recent X-ray analysis) [113] on BPAO by incubation overnight at room temperature in the dark to give a stoichiometric molar labeling ratio of 2 PM per BPAO dimer. The specific activity of the protein was slightly (<13%) decreased by labeling with pyrene maleimide (data not shown).

Figure 37 shows the fluorescence excitation and emission spectra of PM-BPAO incubated in the various denaturants. For all denaturants, the emission spectra showed the characteristic highly resolved vibrational states for monomeric pyrene with the emission peak maxima at 374, 384 and 394nm [173]. In addition, there was no evidence of excimer formation (characterized by a broad peak at 470nm) during either the unfolding or refolding pathways of BPAO using GuHCl, urea or SDS. Therefore, the two pyrene probes attached to the BPAO dimer are spatially distant, which is consistent with the crystal structure location of cysteine 94 residues on each monomer [113].

With respect to native PM-BPAO in Hepes/NaCl buffer, a decrease in the fluorescence emission intensity was observed on incubation with either 5M GuHCl or 8M urea (Figure 37). Thus, with protein unfolding, a quenching of the pyrene maleimide label in BPAO is observed. In contrast, for SDS, addition of 1.73×10^{-2} M SDS resulted in an increase in fluorescence emission intensity for PM-BPAO over that observed in buffer. Furthermore, refolded BPAO in 0.5M GuHCl has a higher intensity than denatured protein (5M GuHCl) but less than the native protein. The changes in fluorescence intensity values are further analyzed in the following section (Section 3.2.2.2.2).

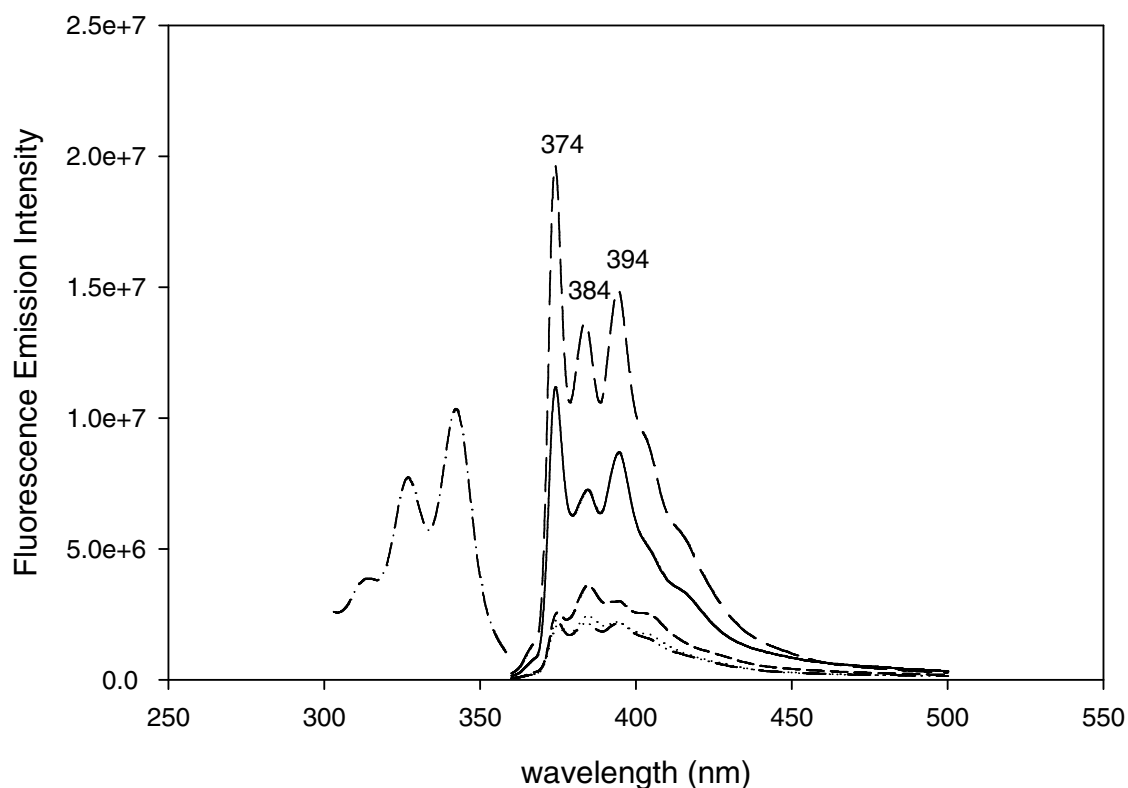


Figure 37: Excitation and emission spectra of pyrene maleimide in PM-BPAO incubated in different denaturant solutions. The concentration of BPAO is $6.06 \pm 0.60 \times 10^{-7} \text{ M}$. The λ_{em} of 377nm was used for the excitation spectrum (---) of BPAO. The maximum peaks in the emission spectra with $\lambda_{\text{ex}} = 344 \text{ nm}$, which represent the vibrational peaks, are shown: 374nm, 384nm and 394nm. The emission spectra lines from top to bottom represent BPAO in 17mM SDS, Hepes buffer, refolded in 0.5M GuHCl, 5M GuHCl and 8M urea. The samples were excited at 344nm with an excitation and emission path length of 3mm, respectively.

3.2.2.2.2 Vibrational Intensity

The fluorescence intensity ratio of vibrational peaks III (384nm) over I (374nm) for pyrene can provide information about the polarity of the probe environment [174, 178, 180]. Specifically, this ratio gives information regarding the immediate environment of the pyrene binding site on BPAO during protein unfolding/refolding or dissociation. Previous studies using solvents of varying polarity have quantified the I_3/I_1 values. An I_3/I_1 value of 0.5 – 0.8 reflects a polar microenvironment, while 1.65 – 1.75 suggests a more nonpolar microenvironment for the pyrene label [178]. Native BPAO labeled with PM molecules showed I_3/I_1 ratios of 0.77 ± 0.17 (Figures 38) which, as expected for Cys 94, is relatively polar. Addition of perturbant may change the ratio of I_3/I_1 as the protein unfolds or dissociates and the resultant environment of the pyrene maleimide label is affected.

While the overall intensity is decreased (Figure 38, *inset*), the addition of GuHCl results in a hyperbolic increase in I_3/I_1 values from 0.77 ± 0.17 to 1.41 (Figure 38). This suggests that with addition of GuHCl, the conformation of BPAO responds so that the PM labels are now more exposed to hydrophobic patches/nonpolar residues in the protein. Refolding experiments show I_3/I_1 values for PM-BPAO consistently higher than observed for denaturation. With dilution from 5M GuHCl, there is an increase to a peak value of 1.81 ± 0.08 observed at 2.25M, followed by a subsequent decrease to 1.48 ± 0.13 as the GuHCl concentration is further diluted to 0.5M. Thus, BPAO does not refold to the native conformation, but rather returns to an alternate stable state. Similar results were proposed from tryptophan intensity data (Figure 29).

In high urea concentrations (9M; Figure 39), there is only a slight increase in I_3/I_1 values for PM-BPAO. It would appear that the polarity of the local environment surrounding the pyrene labels on BPAO does not change significantly with addition of urea.

The I_3/I_1 values for PM-BPAO in SDS (Figure 40) show a decrease ($\Delta = 0.4$), reaching a limiting value of 0.64 with as little as 2.5mM SDS, which is above the cmc of SDS (~1mM) for the buffer conditions used here (0.12M ionic strength [29]). The origins for these data are unclear and suggest that the pyrene labels on the monomers experience a more polar environment with SDS. This may arise because of the micellar character of SDS.

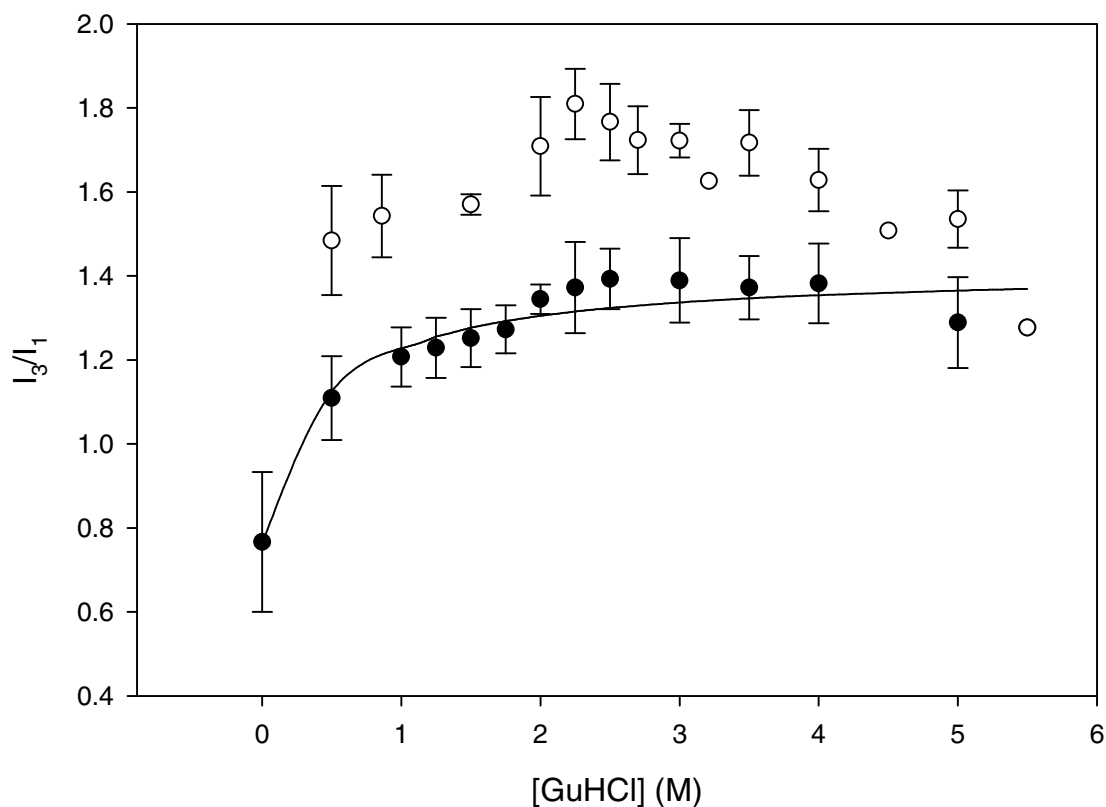


Figure 38: Effect of GuHCl induced denaturation (●) and renaturation (○) on the I_3/I_1 ratio of pyrene maleimide. The line for denaturation is fit to a hyperbola (Equation 68) with a 50% change in I_3/I_1 at 0.40M (●). The concentrations of BPAO in the denatured (●) and renatured (○) samples are $6.06 \pm 0.43 \times 10^{-7}\text{M}$ and $4.23 \pm 1.00 \times 10^{-7}\text{M}$ respectively. The standard deviations reflect 2 (●) and 3 (○) trials. The samples were excited at 344nm with an excitation and emission path length of 3mm, respectively.

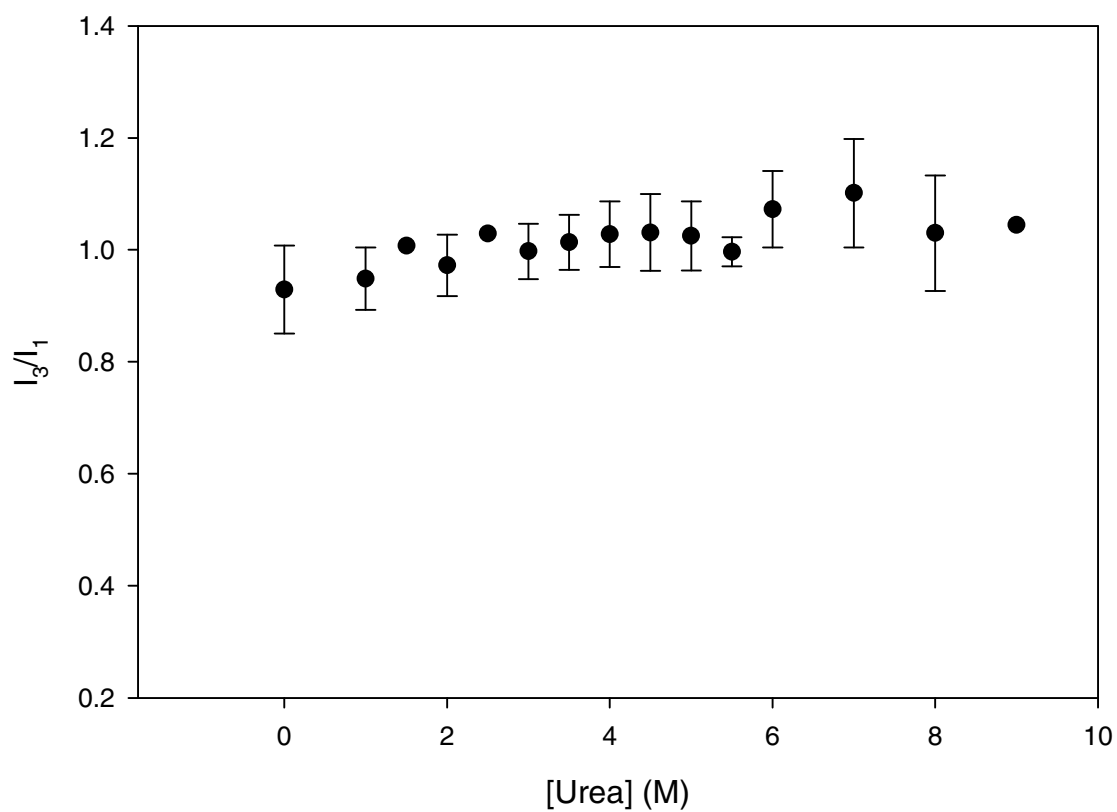


Figure 39: Effect of urea induced denaturation of BPAO on the I_3/I_1 ratio of pyrene maleimide. The concentration of BPAO is $5.38 \pm 0.75 \times 10^{-7} \text{ M}$. The samples were excited at 344nm with an excitation and emission path length of 3mm, respectively. Standard deviations represent 4 trials.

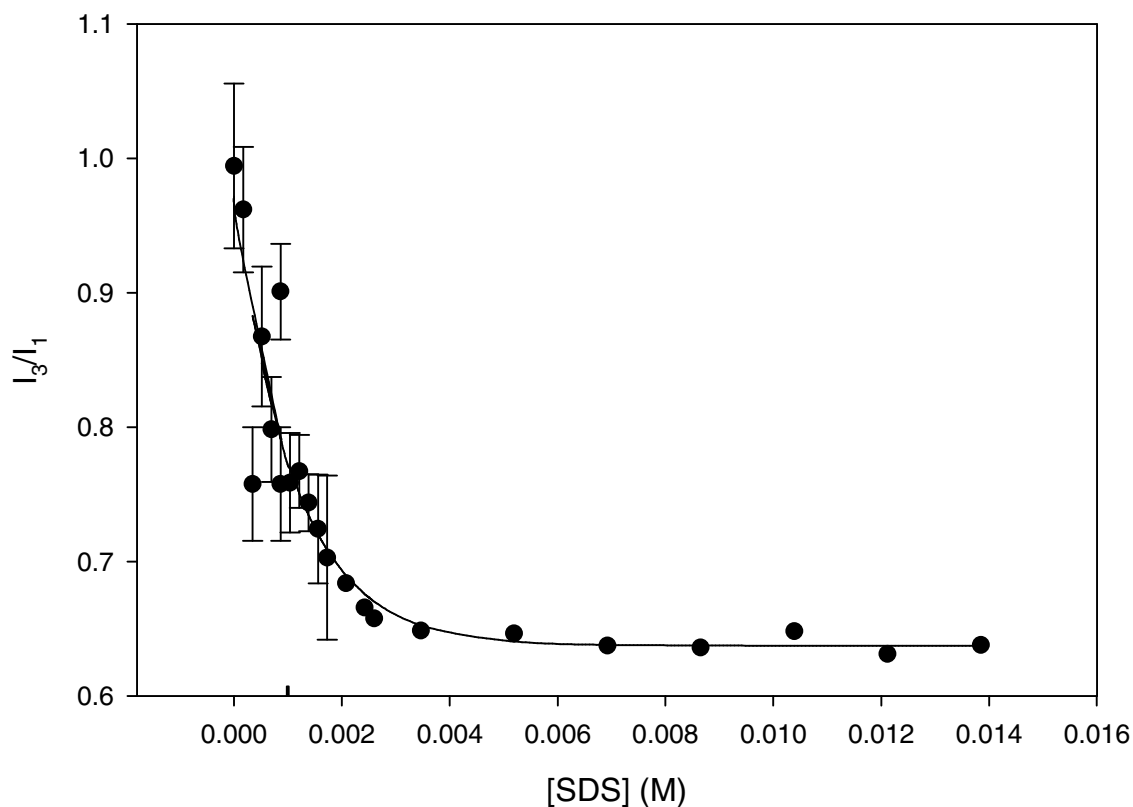


Figure 40: Effect of SDS induced denaturation of BPAO on the I_3/I_1 ratio of pyrene maleimide. The concentration of BPAO is $6.72 \times 10^{-7} \text{M}$. The line represents an exponential decay to 0.64 ($r^2 = 0.8686$). Standard deviations reflect 3 trials and the samples were excited at 344nm with an excitation and emission pathlength of 3mm, respectively. The cmc at 0.12M ionic strength is shown by the vertical line at 1mM SDS [29].

3.2.2.2.3 Steady-State Emission Anisotropy Studies

The fluorescence emission anisotropy, $\langle r \rangle$, is frequently used to monitor the rotations of a fluorophore. For an extrinsic fluorophore such as pyrene maleimide conjugated to a protein, the rotational motions are a combination of those from the global macromolecule, segments of the polypeptide chain and the fluorophore itself. Pyrene maleimide is a unique fluorophore because the long fluorescence lifetime [181-183] offers a time-window sensitive to the slow rotational correlation times reflecting motions of the entire macromolecule. Native BPAO labeled with 2 pyrene maleimide residues per dimer, exhibits steady state emission anisotropy values of 0.146 ± 0.008 (Figures 41, 42 and 43). Pyrene anisotropy studies were undertaken to determine if unfolding and/or dissociation processes for GuHCl treated BPAO could be resolved.

Unfolding of PM-BPAO showed a very distinct and reproducible decrease in emission anisotropy values (from 0.15 to 0.13) with addition of low GuHCl concentrations (0.5M) (Figure 41). As the concentration of GuHCl was further increased to 5M, the emission anisotropy undergoes a sigmoidal decrease to 0.09 with a transition midpoint at 2.5M, before decreasing slightly to 0.07 in 5.5M. This data suggests a dissociation of BPAO in GuHCl. While the anisotropy data were not corrected for lifetime changes, the overall observed trends would be the same, since the normalized total fluorescence intensity (Figure 41, *inset*) shows an exponential decrease of about 75% with 5.5M GuHCl as the protein is perturbed. The origins for the decrease are unclear but may result from a change in the lifetime of the PM fluorophore due to dynamic quenching (Equation 20) by GuHCl.

Refolding experiments (Figure 41) show a sigmoidal increase in emission anisotropy as BPAO is refolded by dilution from 5M to 0.5M GuHCl with a transition midpoint of 2.6M. The normalized total intensity (Figure 41, *inset*) does not change upon refolding and is noticeably less than those intensity values obtained for the perturbed protein. It is noteworthy that while the emission anisotropy values at GuHCl concentrations between 0.5 to 2M for both the perturbed and renatured conformations are the same, the normalized total intensities are very different which suggests that upon renaturation, the environment of the fluorophores represents an alternative initial state. Similar results were obtained from tryptophan fluorescence studies (Figure 29) and pyrene maleimide intensity (Figure 38) results.

There is no significant change in emission anisotropy unless for PM-BPAO with the addition of up to 4.25M urea (Figure 42). At urea concentrations greater than 4.25M there is a decrease from 0.143 to 0.110 at 8M. Since no sigmoidal transition (Equation 67) was observed, this suggests partial dissociation of the BPAO homodimer. The plot of normalized total intensity (Figure 42, *inset*) shows a decrease (~50%) as the concentration of urea is increased, which may reflect changes in the lifetime of the fluorophores (Equation 19).

Figure 43 shows the effect of SDS on the anisotropy of PM-BPAO for three different protein concentrations (0.1, 2.24 and 6.7 μ M). The concentration of SDS necessary to monitor the change in PM anisotropy was much lower than used for previous (tryptophan) spectroscopic studies (Figure 31). A sigmoidal decrease in emission anisotropy values from 0.14 to 0.06 was observed, with midpoints at 0.76, 0.94 and 0.87 mM SDS, respectively, for each protein concentration used. This emission

anisotropy decrease strongly suggests dissociation of the BPAO homodimer from a dimer (0.14) to monomers (0.06) by SDS. The transition midpoints are equivalent to the cmc of SDS (~1mM) [29] and suggest that micelles may interact with the BPAO dimer and lead to the dissociation into monomers. The normalized total intensity for SDS with PM-BPAO (Figure 31, *inset*) exhibits hyperbolic characteristics with a 2-fold increase and a dissociation constant of 0.173mM SDS (Figure 43, *inset*).

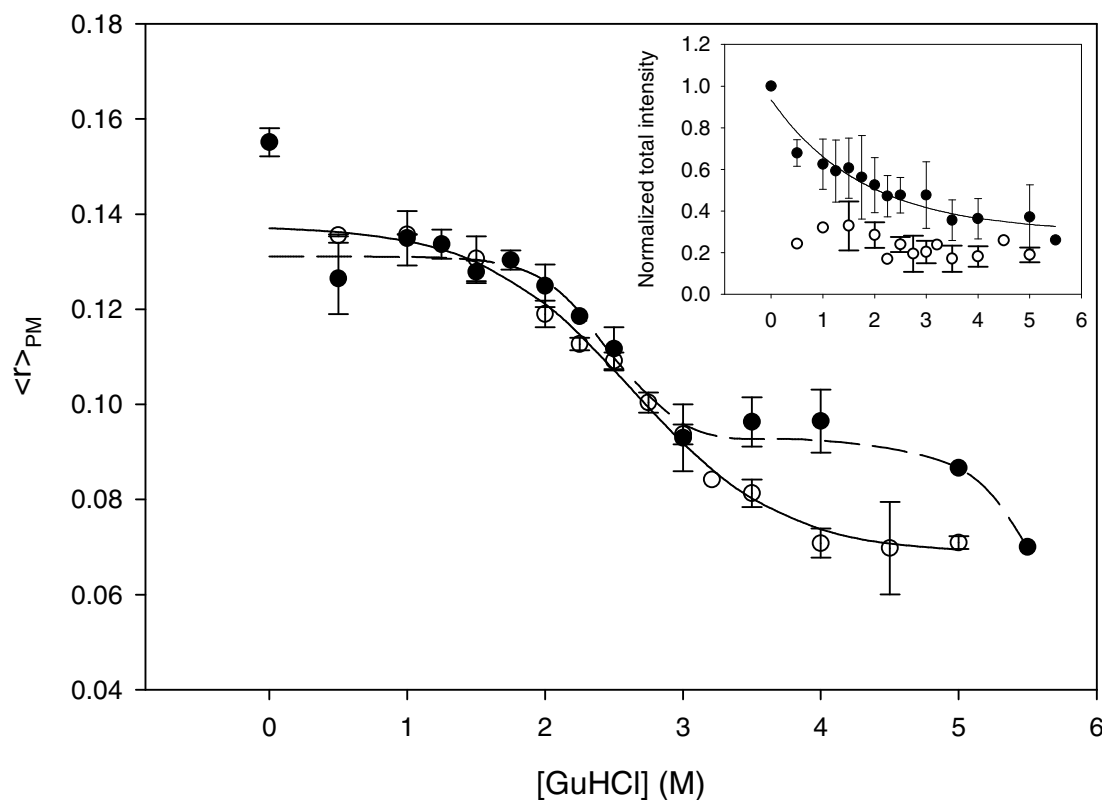


Figure 41: The effect of GuHCl on steady-state pyrene anisotropy of PM-BPAO. The concentrations of denatured ($\bullet$) and renatured ($\circ$) BPAO are $6.06 \pm 0.43 \times 10^{-7} \text{ M}$ and $4.23 \pm 1.00 \times 10^{-7} \text{ M}$, respectively. The lines represent the sigmoidal fit of the data (Equation 67; with the exception 0 and 5.5M data points for denaturation) with transition midpoints at 2.45M ($\bullet$) and 2.63M ($\circ$) GuHCl. *Inset:* The corresponding total intensity (Equation 24) normalized to 0M GuHCl. The line is shown only to illustrate the trend. The standard deviations represent 2 denatured and 3 renatured trials.

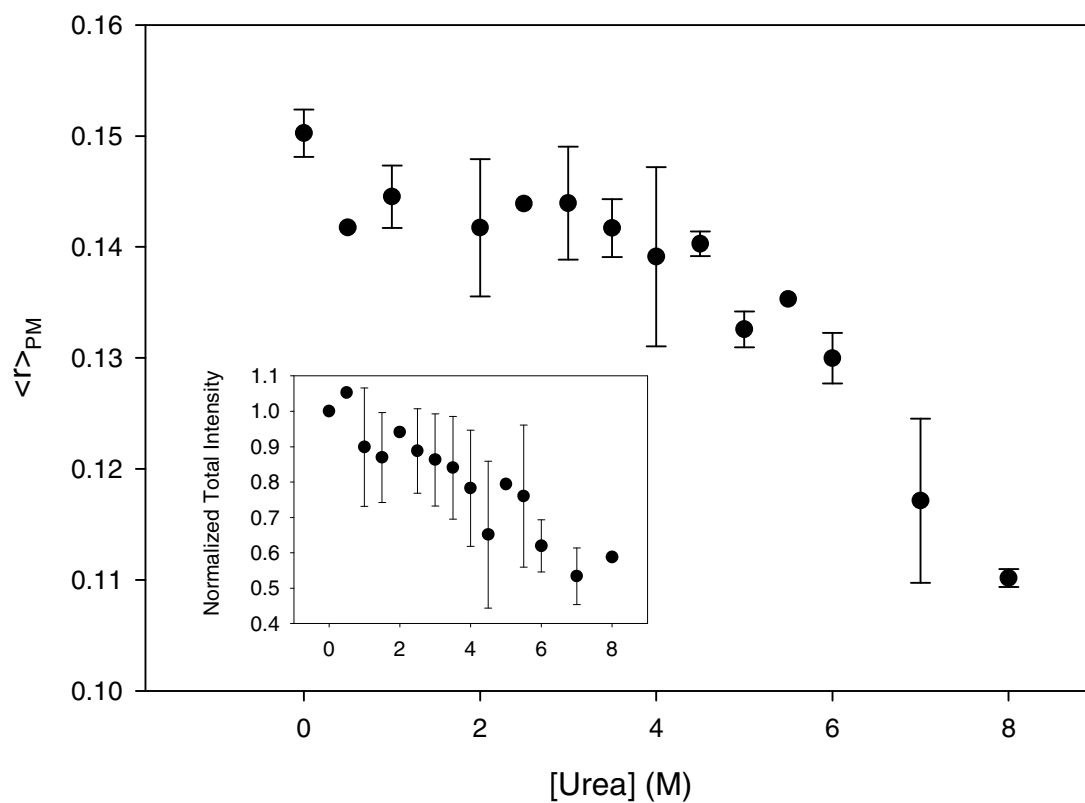


Figure 42: The effect of urea on steady-state pyrene emission anisotropy of PM-BPAO. The concentration of BPAO is $5.38 \pm 0.75 \times 10^{-7} \text{ M}$. The standard deviations reflect 4 trials. *Inset*: The corresponding total intensity calculated by Equation 24, was normalized to 0M urea.

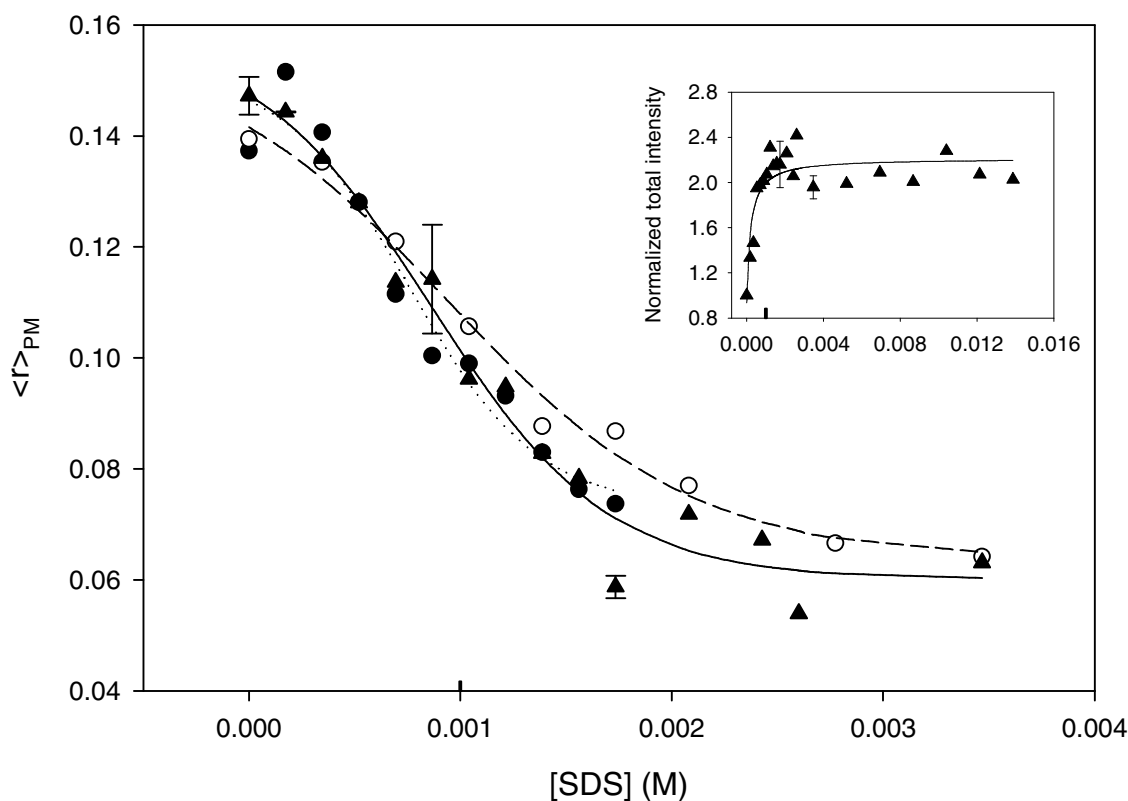


Figure 43: The effect of SDS on the steady-state pyrene emission anisotropy of PM-BPAO. The concentrations of denatured BPAO are $1.11 \times 10^{-7} \text{ M}$ (●), $2.24 \times 10^{-6} \text{ M}$ (○) and $6.72 \times 10^{-6} \text{ M}$ (▲). The lines represent a sigmoidal fit to the data (Equation 67) with transition midpoints at $7.6 \times 10^{-4} \text{ M}$ (●), $9.4 \times 10^{-4} \text{ M}$ (○) and $8.7 \times 10^{-4} \text{ M}$ (▲) SDS, respectively. The cmc in 0.12M ionic strength is shown by the vertical line at 1mM SDS [29]. *Inset*: The corresponding total intensity calculated by Equation 24 for $6.72 \times 10^{-6} \text{ M}$ BPAO (▲), normalized to 0M SDS. The line shows the hyperbolic increase (Equation 68) with a 50% increase in total intensity at $1.73 \times 10^{-4} \text{ M}$ SDS and r^2 of 0.7717. The standard deviations result from 2 trials.

3.2.2.2.4 Time-Resolved Fluorescence Lifetime Studies

In addition to steady-state fluorescence intensity measurements, time-resolved fluorescence lifetime studies can also provide valuable information about the global state of a protein (Section 1.5.1.4.1). Figure 44 shows the intensity decay for pyrene maleimide labeled BPAO. The fluorescence decay was fit to the sum of three exponentials and the resolved lifetime values were 38.8ns, 8.65ns and 1.97ns (Table 12). The pre-exponential factors (α) associated with the two shorter lifetimes (0.68, 0.59) are much greater than those for the long lifetime ($\alpha_1=0.11$).

The addition of GuHCl to PM- BPAO resulted in a change in the pyrene maleimide lifetimes (Figure 45, Table 12). The long (τ_{long}) and medium (τ_{short}) lifetimes both show sigmoidal decreases to 24.4 and 4.81ns with transition midpoints at 2.88 and 3.22M GuHCl respectively. The shortest lifetime (τ_{short}) does not change appreciably compared with the other lifetime values.

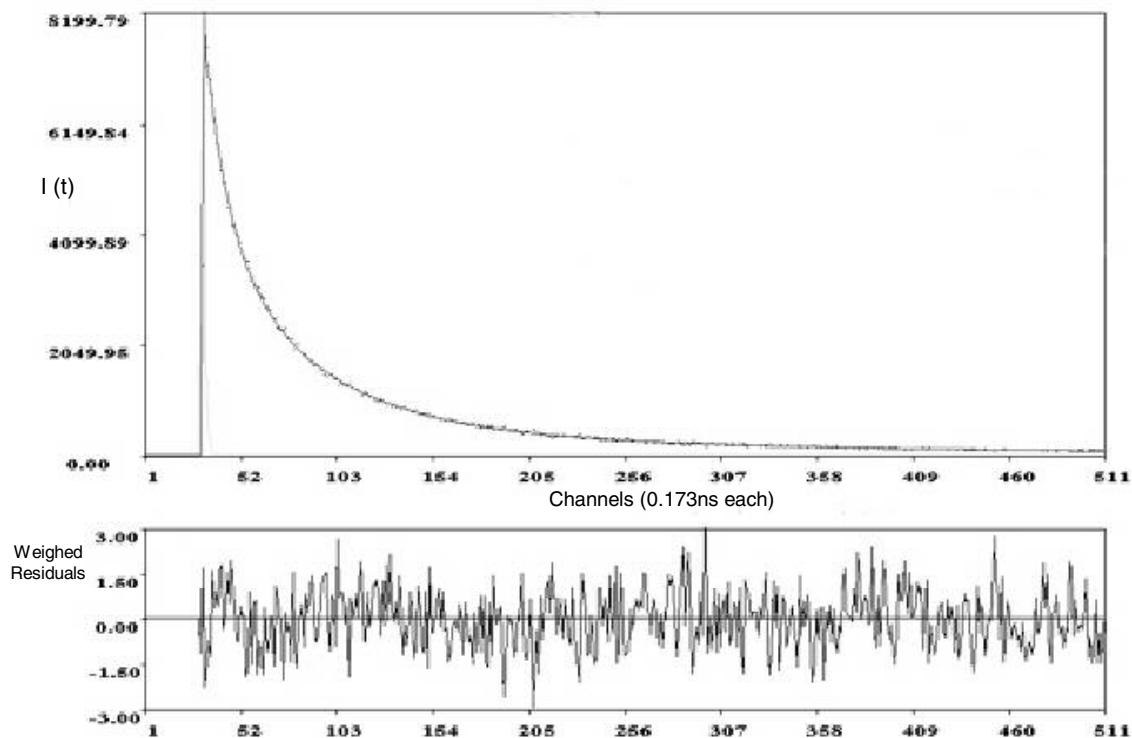


Figure 44: Time resolved fluorescence emission intensity of BPAO labeled with pyrene maleimide. (A) The line is the fit to the intensity decay as a function of the number of channels. (B) Weighted residuals of the intensity decay fit to the sum of three exponentials ($\chi^2 = 1.022$). Excitation is at 335nm and emission is collected at 420nm. The concentration of BPAO is 7.5×10^{-7} M in 0.02M Hepes and 0.1M NaCl pH 7.5 buffer at 28°C.

Table 12:
Parameters Associated with the Fluorescence Intensity decay of BPAO at 28°C^a.

[GuHCl] (M)	α_{long}	α_{medium}	α_{short}	τ_{long}	τ_{medium}	τ_{short}	χ^2
0	0.108	0.677	0.589	38.8	8.65	1.97	1.022
1	0.115	0.718	0.725	37.8	9.13	2.21	1.151
2.5	0.087	0.525	0.651	33.6	8.29	2.16	1.095
3.5	0.068	0.515	0.640	28.5	6.63	2.00	1.233
5	0.076	0.647	0.711	24.4	4.80	1.18	1.059

^a $\tau_{\text{long}} - \tau_{\text{short}}$ (ns) are the fluorescence lifetimes and $\alpha_{\text{long}} - \alpha_{\text{short}}$ are the corresponding relative amplitudes (Equation 56).

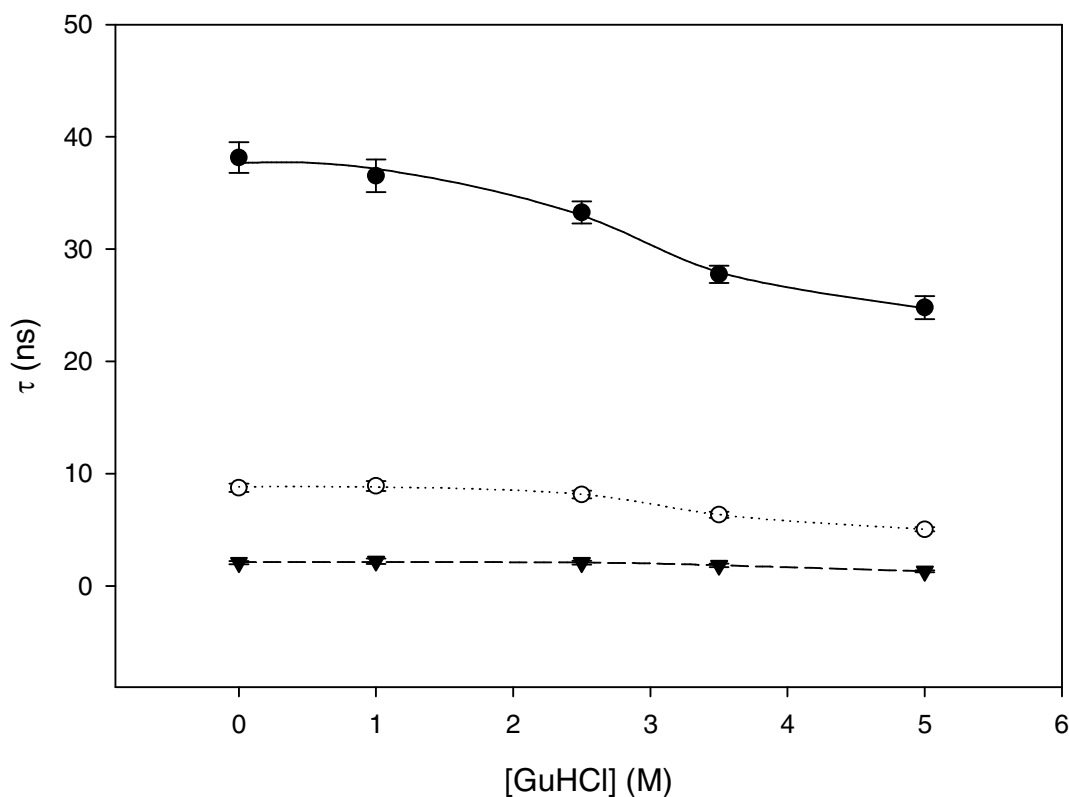


Figure 45: Fluorescence lifetime values for pyrene maleimide *versus* GuHCl concentration. The lines represent a sigmoidal fit to the data (Equation 67). The long (●) and medium (○) lifetimes show transition midpoints at 2.88M and 3.22M GuHCl, respectively. The short lifetime (▼) does not change considerably. The concentration of BPAO is $8.25 \pm 1.06 \times 10^{-7}$ M. Laser excitation is at 335nm and emission is monitored at 420 nm. The standard deviations shown for all lifetime components reflect at least 3 trials.

3.2.2.2.5 Time resolved emission anisotropy (TREA)

TREA studies can provide more detailed information about the underlying rotational processes for a complex system than reported by steady-state studies. In particular, time dependent studies provide information about the contributions from each of the underlying rotational components. Such analyses can aid in understanding the conformational changes and dissociation properties of a protein. Iterative non-linear deconvolution methods were used to recover the rotational correlation times (ϕ_j) and their associated pre-exponential values (β_j) from the multiexponential polarized decays (Section 2.2.5.2.1).

For GuHCl/PM-BPAO solutions, the TREA decay profiles were fit using a double or triple exponential decay function depending on the concentration of GuHCl used. For GuHCl concentrations less than or equal to 2.5M, the best fit to the anisotropy decay for PM-BPAO was achieved using three exponentials (ϕ_{long} , ϕ_{medium} and ϕ_{short}). For GuHCl concentrations greater than 2.5M, the best fit was a biexponential function (ϕ_{medium} and ϕ_{short} , Table 13).

A summary of the data fits in terms of the retrieved rotational correlation times (ϕ_j) and pre-exponential terms (β_j) are shown in Figure 46 and Table 13. The long rotational correlation time ($\phi_{\text{long}} = 164\text{ns}$) suggests rotational motions of the entire homodimeric protein. On addition of 2.5M GuHCl, a small decrease in the rotational correlation time (from 164ns to 108ns) and their associated amplitudes (from 0.15 to 0.12) was observed. Interestingly the slow rotational motions (ϕ_{long}) could only be resolved up to the addition of 2.5M GuHCl and was not present at GuHCl concentrations greater than 2.5M. Other retrieved rotational components, ϕ_{medium} and ϕ_{short} , suggest

local protein segmental motions (9 – 17ns) and fast rotations of the PM fluorophore itself (0.4 - 0.8ns) [259]. The pre-exponential terms (partial anisotropy) associated with the medium (β_{medium}) and short (β_{short}) rotational components are much smaller than associated with the long component (β_{long}) in the range of 0 - 2.5M GuHCl.

At GuHCl concentrations greater than 2.5M, the rotational picture for PM-BPAO is changed. A sharp increase in both β_{short} and β_{medium} (Figure 46, Table 13) is observed with a concomitant decrease in β_{long} . In addition, the slow rotational correlation times (ϕ_{long}) decreases dramatically, and the rotational decay for PM-BPAO is now dominated by molecular motions of a faster rotating species, assumed to arise from a monomeric species and that of the fluorophore itself. These data suggest that the protein conformation alters in such a manner that the local rotations of the monomer and fluorophore are increased considerably at GuHCl concentrations over 2.5M, whereas little rotational information on the BPAO dimer can be resolved. This suggests dissociation of the homodimeric form of BPAO.

Molecular weights may be estimated from the experimentally determined rotational parameters (Stokes-Einstein Equation 26) and are summarized in Table 14. Values were determined from rotational correlation times that were corrected for changes in the viscosity of GuHCl solutions [248]. The calculated molecular weight value for the native protein (491kD, using $\phi_{\text{long}} = 164\text{ns}$) is 2.5 times larger than the expected for a protein of 200kD. Discrepancies may arise due to the non-spherical conformation of the protein [113]. It may also reflect the highly glycosylated nature of the BPAO dimer [113, 260] and associated water of hydration layer. Assuming a sphere for BPAO, the radius calculated for a rotational correlation time of 164ns is 56.6Å using the Stokes-Einstein

equation (Equation 26) and the volume of a sphere ($V = \frac{4}{3}\pi r^3$). Previous studies with pig PAO report a rotational correlation time of 120ns with two pyrenebutyric anhydride molecules bound per dimer [233]. The medium rotational correlation time (ϕ_{medium}) shows a maximum value at 3.5M GuHCl which corresponds to a molecular weight of 138kD. This suggests dissociation of homodimeric BPAO under these conditions.

The fractional contribution of the individual rotational correlation times ($f_{\beta_j\phi_j}$; Equation 61) to the anisotropy decay as a function of GuHCl is shown in Figure 47. With increasing GuHCl concentrations, the long component ($f_{\beta_{\text{long}}\phi_{\text{long}}}$) decreases with a concomitant increase in the medium one ($f_{\beta_{\text{medium}}\phi_{\text{medium}}}$). The long component clearly reflects dissociation of the dimer while the medium component reflects formation of monomeric species.

Steady state emission anisotropy values for PM-BPAO ($\langle r \rangle_{\text{TR}}$) can be back calculated from the time resolved polarized and lifetime data and are illustrated graphically in Figure 48 along with previous steady state values ($\langle r \rangle_{\text{SS}}$, Section 3.2.2.2.3). In the absence of added GuHCl, the values of emission anisotropy from time-resolved and steady-state measurements are similar (0.153). In contrast to steady-state measurements, emission anisotropy values from time-resolved studies ($\langle r \rangle_{\text{TR}}$) remain unchanged with addition of 1M GuHCl. Further, the transition midpoint is shifted to higher GuHCl concentrations for time resolved derived anisotropy values ($C_m \sim 3M$) as compared with steady state measurements ($C_m = 2.45M$; Figure 41). Indeed for each [GuHCl], $\langle r \rangle_{\text{TR}}$ is greater than $\langle r \rangle_{\text{SS}}$. The origins for the discrepancies are unclear, however, they may be derived from truncation errors due to the long fluorescence decay

profile for pyrene, or that the decay analysis of the time-resolved emission anisotropy profile requires greater than three components.

Table 13:
Experimental Parameters Associated with the Pyrene Maleimide Fluorescence
Anisotropy Decay of PM-BPAO at 28°C^a

[GuHCl] (M)	β_{long}	β_{medium}	β_{short}	ϕ_{long}	ϕ_{medium}	ϕ_{short}	r_0^b	χ^2^c
0	0.146	0.0318	0.0386	163.8	9.094	0.393	0.217	1.027
1	0.139	0.0424	0.0292	192.7	15.06	0.626	0.210	1.024
2.5	0.123	0.0278	0.0705	107.6	30.18	0.683	0.222	1.222
3.5	0	0.1195	0.2050	0	38.66	1.505	0.325	1.409
5	0	0.0860	0.2052	0	17.33	0.750	0.291	1.100

^a ϕ is the observed rotational correlation time measured in ns and β is the corresponding amplitude. 0, 1 and 2.5M GuHCl data are fit to three exponential terms while the best fit for 3.5 and 5M GuHCl are double exponentials.

^b Initial anisotropy (r_0) calculated by Equation 31.

^c χ^2 is the difference between the original data and the calculated fit.

Table 14:
Determination of molecular weight (kD) from the corrected rotational correlation times,
 ϕ_{long} and ϕ_{medium} (ns), of pyrene maleimide anisotropy of PM-BPAO at 28°C ^a.

[GuHCl] (M)	ϕ_{long}	MW _{long}	ϕ_{medium}	MW _{medium}
0	164	491	9	27
1	200	599	16	48
2.5	122	365	34	101
3.5	0	0	46	138
5	0	0	24	72

^a Calculated from the Stokes-Einstein equation (Equation 26).

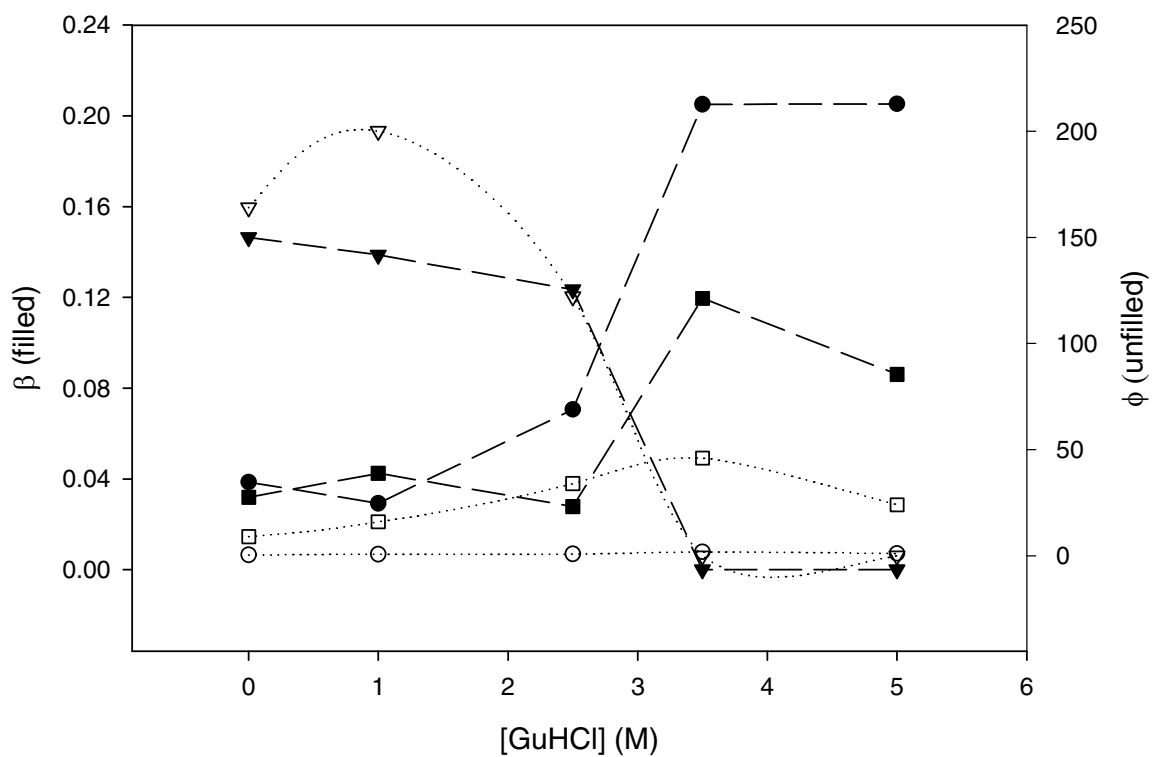


Figure 46: The effect of GuHCl on the rotational properties of pyrene maleimide labeled BPAO. The long (▼, ▽), medium (●, ○) and short (■, □) viscosity corrected rotational correlation time, ϕ (unfilled symbols), and amplitude, β (filled symbols). The concentration of BPAO is $8.25 \pm 1.06 \times 10^{-7} \text{ M}$. The lines reflect the trend in ϕ (dotted) and β (dashed). Excitation was at 335nm and emission was monitored at 420nm.

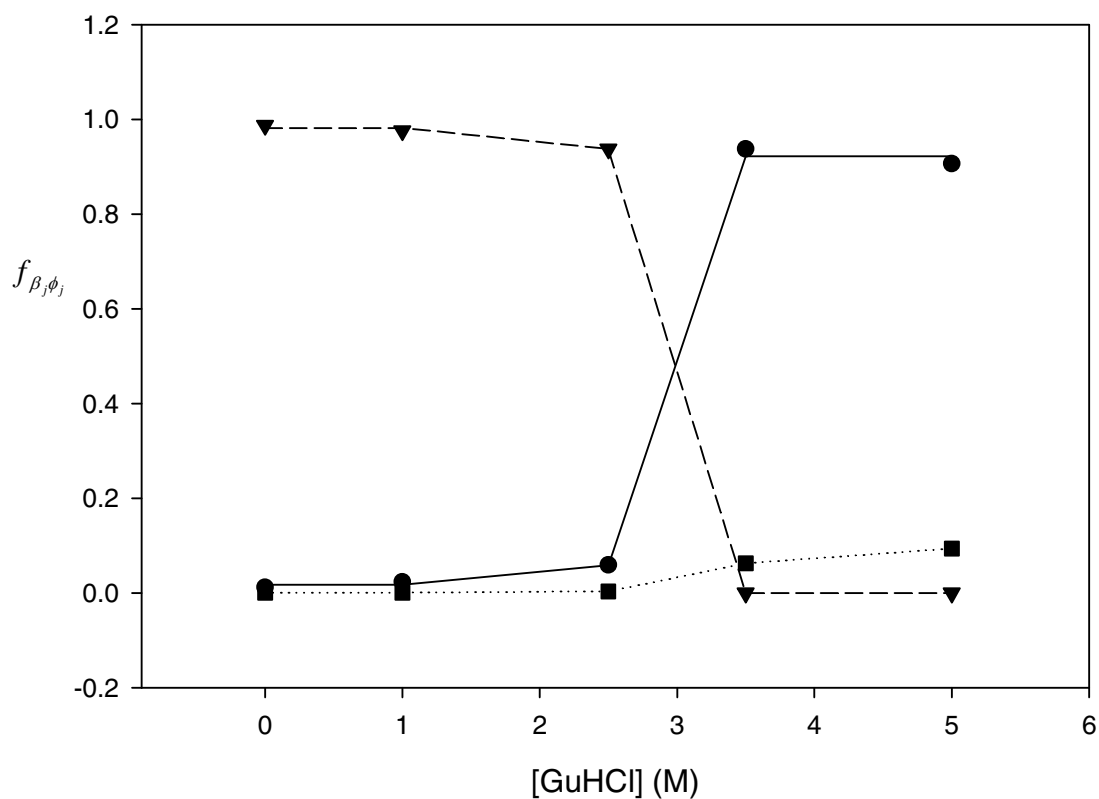


Figure 47: The effect of GuHCl on the fractional anisotropy contribution of pyrene maleimide labeled BPAO. The concentration of BPAO is $8.25 \pm 1.06 \times 10^{-7} \text{ M}$ for long (▼), medium (●) and short (■) fractional anisotropy contribution. The lines reflect the sigmoidal trend (Equation 67) with midpoint transitions at 2.75 (▼), 2.69 (●) and 3.34M (■) GuHCl. Excitation was at 335nm and emission was monitored at 420nm. Fractional emission anisotropy is calculated by Equation 61.

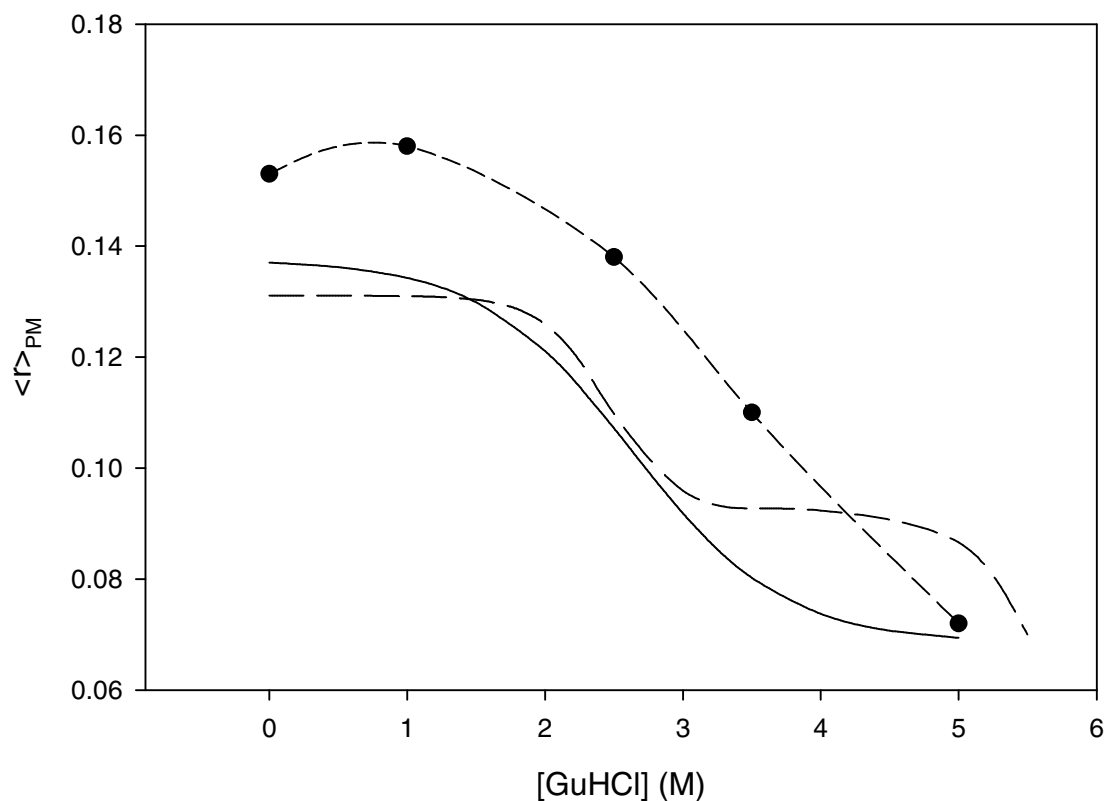


Figure 48: The effect of GuHCl on the time resolved anisotropy of PM-BPAO (-●-). Steady-state anisotropy (Equation 33) for denaturation (dashed line) and renaturation (solid line) represent the sigmoidal fit to the data (Figure 46). The concentration of BPAO is $8.25 \pm 1.06 \times 10^{-7} \text{ M}$ for time resolved studies and $6.06 \pm 0.43 \times 10^{-7} \text{ M}$ (denaturation) and $4.23 \pm 1.00 \times 10^{-7} \text{ M}$ (renaturation) for steady-state experiments. Excitation was at 335nm and emission was monitored at 420nm for time resolved data. For steady state data, excitation and emission were monitored at 344 and 377nm, respectively.

3.2.3 High Pressure Liquid Chromatography

HPLC is another technique that can provide information about the global conformation (dimer-dissociation) of a protein. SEC-HPLC is useful for studying protein folding/unfolding due to its ability to resolve changes in the hydrodynamic volumes of macromolecules. The Stokes radius (R_s) of a protein is calculated directly from the elution volume [261]. HPLC can detect both kinetic intermediates that are stable within the time scale of the chromatographic run [87, 262-264] or protein aggregation [265].

HPLC analyses of BPAO in GuHCl were performed to provide further insights into the unfolding and dissociation pathways. Figure 49 shows the elution chromatogram for purified BPAO in Hepes buffer with 0.1M NaCl. A predominant peak was observed at a retention time of 8.81min, corresponding to an elution volume of 2.64ml (Equation 34) using a 0.3ml/min flow rate, and was attributed to the dimeric form of BPAO. In addition, a low concentration of high molecular weight aggregates was observed (Peak A). From the calibration curve for molecular weight *versus* retention time (Equation 68, Section 2.2.6), the calculated molecular weight for the native BPAO peak by HPLC was determined to be 192kD, which provides a corresponding Stokes radius for the native BPAO dimer of 55.4Å. This correlates well with the value of 56.6Å determined from time resolved data (Section 3.2.2.2.5).

With addition of low concentrations of GuHCl (0.5M), an initial increase in the elution volume of dimeric BPAO (2.78 ± 0.04 ml) was observed which suggests the possible formation of a compact intermediate (Figure 50). A similar effect was observed from steady-state emission anisotropy studies of PM-BPAO (Figure 41) at low GuHCl concentrations and is attributed to protein “shrinking” caused by the binding of GuHCl

ions [264] to the BPAO dimer. With increasing denaturant concentrations to 5M GuHCl, a sigmoidal decrease in elution volume from 2.78ml to 2.26 ± 0.01 ml was observed with a transition midpoint at 2.23 ± 0.01 M. Interestingly, there is no evidence of a monomeric peak at 9.62min (corresponding to an elution volume of 2.89ml, calculated by Equation 64 and 66 using a molecular weight for the monomer of 96kD) even in 5M GuHCl (Figure 51). Renaturation of BPAO by dilution from 5M GuHCl follows the denaturation pattern, with the transition midpoint occurring at 2.25M (Figure 50). Therefore, it would appear from HPLC analyses, that the hydrodynamic properties of the denatured and renatured conformations are similar, in contrast to tryptophan fluorescence intensity studies (Sections 3.2.2.1.1).

The Stokes radii were determined from calibration of the column using standards of known molecular weight (Figure 52). As the concentration of GuHCl increases from 0.5 to 5M, the Stokes radii increase sigmoidally from 46 to $95 \text{ \AA}$ which suggests unfolding of the homodimeric BPAO to a conformation that corresponds to a species with twice the radius of the native protein.

We sought to rationalize the lack of a monomeric peak in the elution profile (Figure 49) given that the pyrene maleimide emission anisotropy results suggest that the addition of GuHCl leads ultimately to the dissociation of the homodimeric protein following the initial dimeric unfolding (Sections 3.2.2.2.3 and 3.2.2.2.5). We propose that HPLC is actually reporting on the dissociation of the dimer into unfolded (M_U) rather than folded (M) monomers. As the monomer unfolds, the hydrodynamic radius, not molecular weight, increases greater than that for the dimer. This phenomenon where the

dimer dissociates directly into the unfolded monomers has also been reported for dimeric glutathione S-transferase [87].

For HPLC studies of the urea promoted unfolding of BPAO measured by HPLC, the elution volume remained constant with the addition of up to 5M urea after which there was a decrease in elution volume to 2.32ml with addition of 8M urea (Figure 53). As with GuHCl, there is no peak at 9.62min (2.89 ml) corresponding to a predicted monomeric BPAO. Determination of the Stokes radii from the elution volume suggests an increase to 88Å in 8M urea from 55 Å in buffer (Figure 54). This suggests possible dimeric unfolding as was proposed from UGGE studies (Figure 26). The pyrene maleimide emission anisotropy studies (Figure 42) suggests partial dissociation of the homodimer into monomers at higher urea concentration. Therefore, as with GuHCl above, analysis of all results reflect formation of an unfolded monomeric conformation (M_U) with an expanded hydrodynamic size.

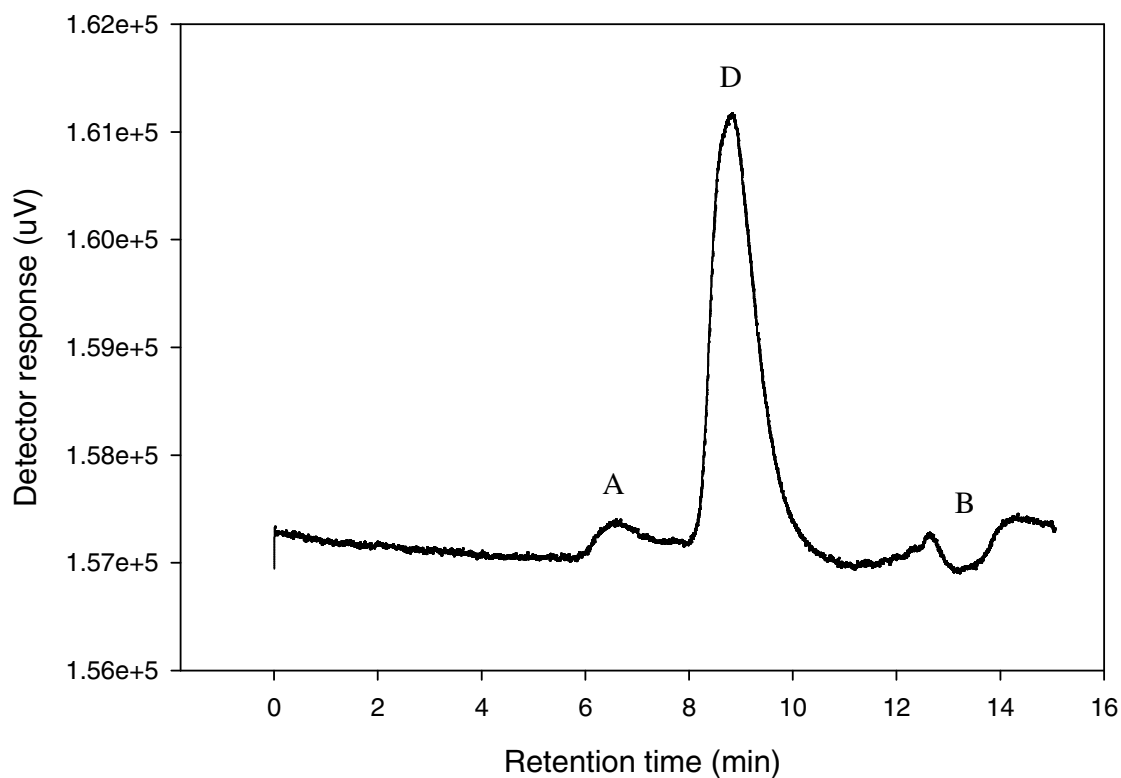


Figure 49: Elution chromatogram for BPAO. The solvent is 0.02M Hepes and 0.1M NaCl, pH 7.5. The flow rate is 0.3ml/min and protein elution is measured spectrophotometrically at 255nm. The first peak (A) at 6.40min represents aggregates and the second peak (D) at 8.81min represents the purified dimer. B represents a bubble. The concentration of BPAO is 2.5×10^{-7} M.

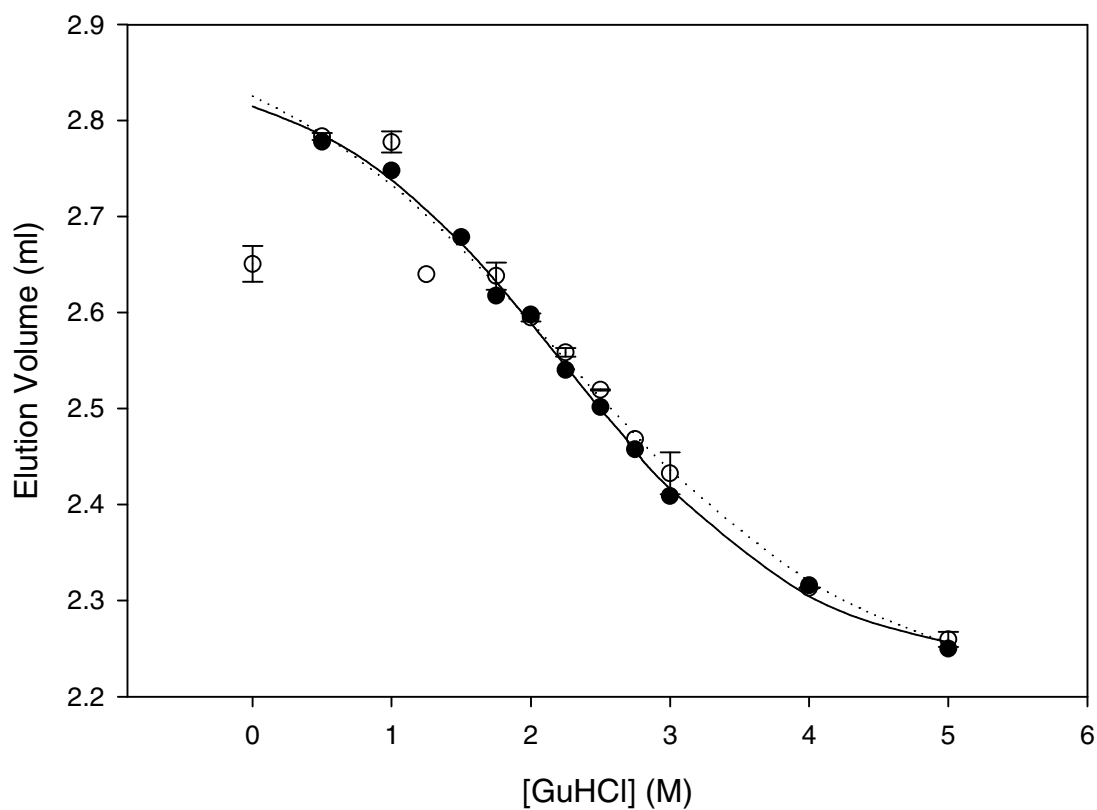


Figure 50: The effect of GuHCl on the elution volume of BPAO. The concentration of BPAO denatured (○) and renatured (●) is 2.5×10^{-7} M. Absorbance is measured at 255nm with a flow rate of 0.3ml/min. The lines represent the sigmoidal fit to the data excluding the native data (Equation 67) and the transition midpoint (C_m) for denatured and renatured BPAO is 2.23 ± 0.01 and 2.25M GuHCl, respectively. The average of two trials is shown for the denatured protein.

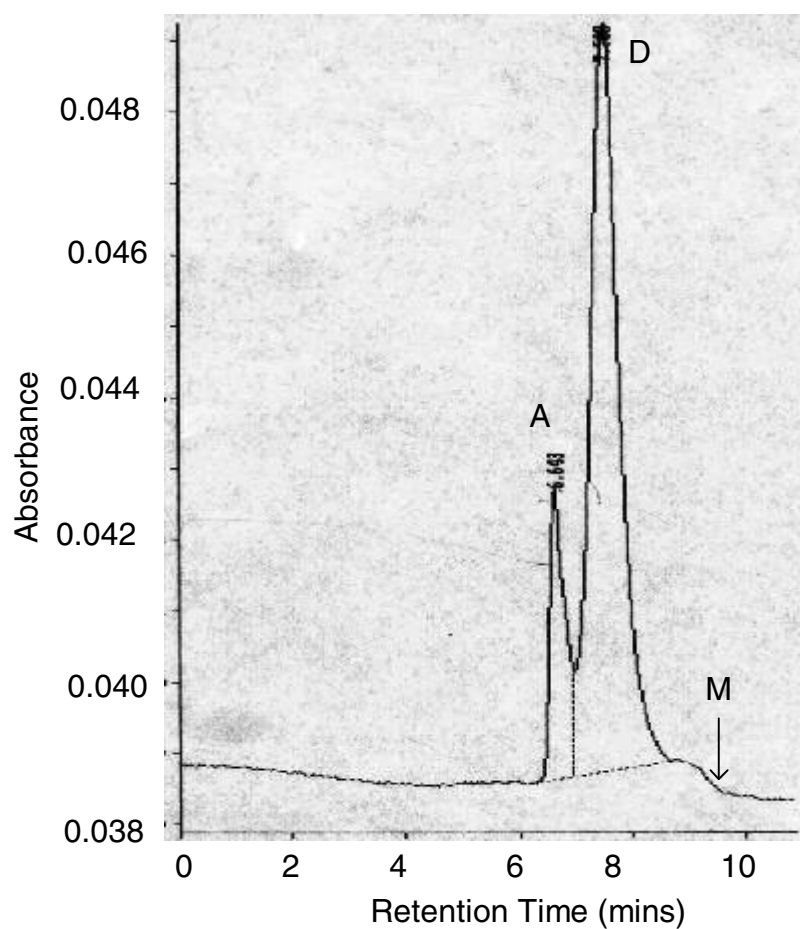


Figure 51: Elution chromatogram for BPAO in 5M GuHCl. The flow rate is 0.3ml/min and protein elution is measured from the absorbance at 255nm. The first peak (A) at 6.64min represents aggregates and the second peak (D) at 7.50min represents the denatured dimer. M shows the monomeric peak at 9.62min expected for dissociation. The concentration of BPAO is 2.5×10^{-7} M.

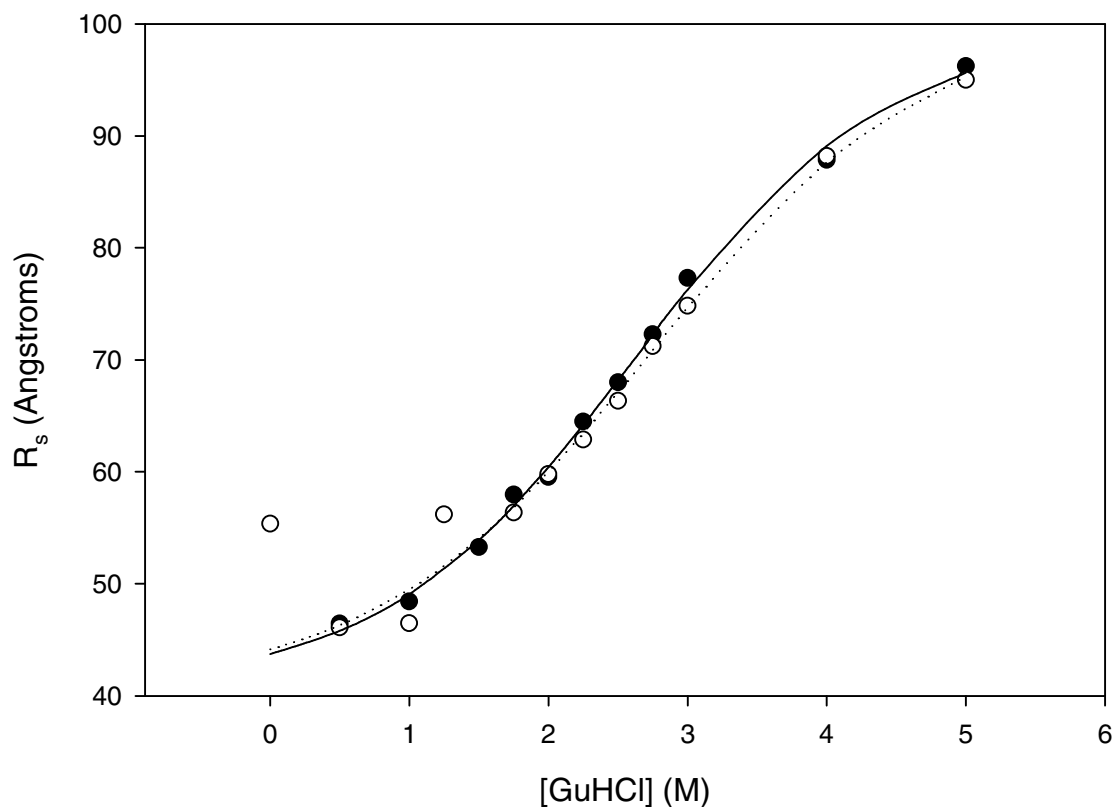


Figure 52: The effect of GuHCl on the Stokes Radii of BPAO measured by HPLC. The concentration of BPAO denatured (○) and renatured (●) is 2.5×10^{-7} M. Absorbance is measured at 255nm with a flow rate of 0.3ml/min. The lines represent the sigmoidal fit to the data (Equation 67) excluding the native data point. The transition midpoint for denatured and renatured BPAO is 2.74 and 2.62M GuHCl. Stokes radii is calculated from a calibration curve (Equation 65).

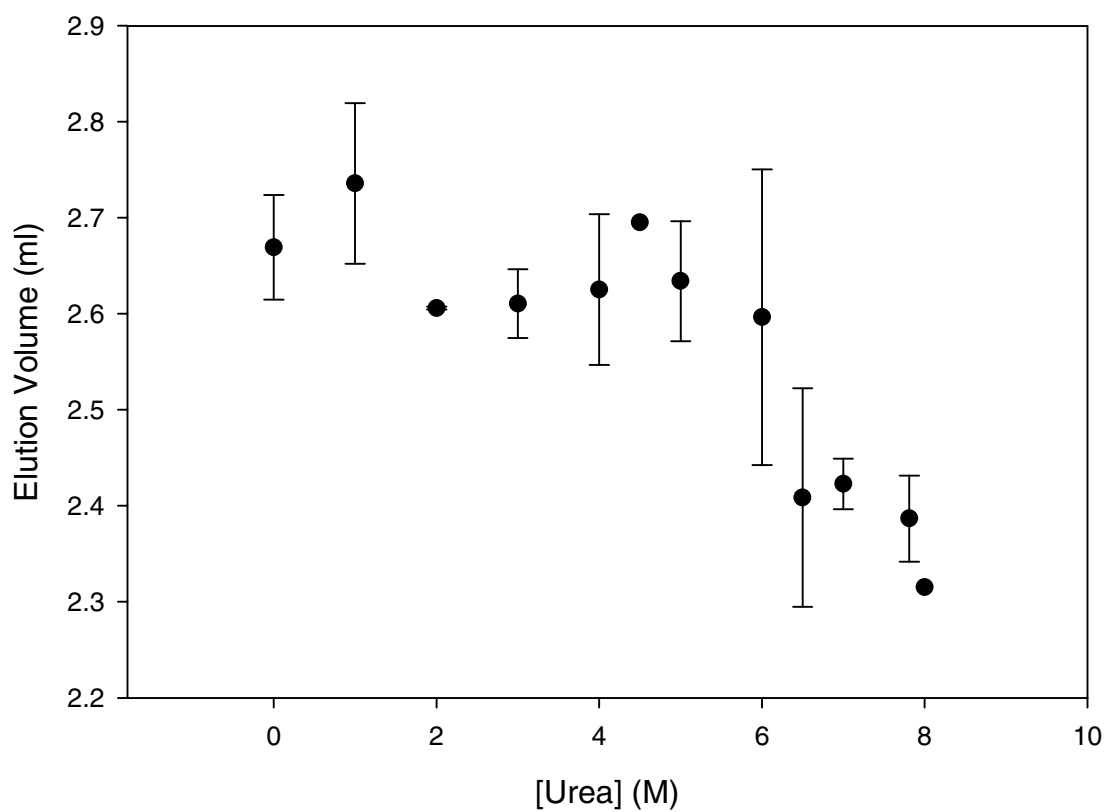


Figure 53: The effect of urea on the elution volume of BPAO. The concentration of denatured BPAO (●) is 2.5×10^{-7} M. Absorbance is measured at 255nm with a flow rate of 0.3ml/min. The standard deviations reflect at least 3 trials.

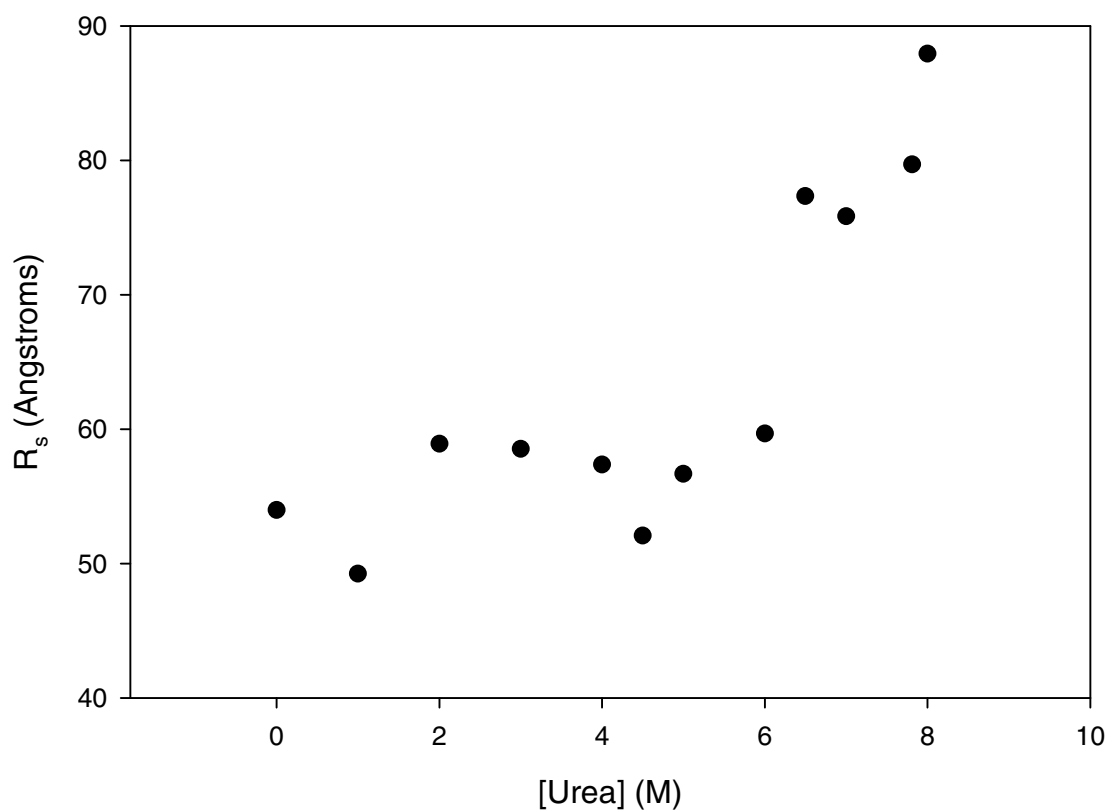


Figure 54: The effect of urea on the Stokes Radii of BPAO measured by HPLC. The concentration of denatured BPAO (●) is 2.5×10^{-7} M. Absorbance is measured at 255 nm with a flow rate of 0.3 ml/min. Stokes radii is calculated from a calibration curve (Equation 65).

3.3 Presence of intermediates

As has been observed for other proteins [16, 20-23], we reasoned that during unfolding of BPAO, hydrophobic sites may be exposed and molten globule intermediates may form. Thus, during the perturbation of BPAO with urea and GuHCl, studies using fluorescent probes that bind to and are sensitive to hydrophobic patches on a protein were used to determine the existence of molten globule intermediate conformations.

The molten globule intermediate has conformational characteristics of both the native and unfolded protein (Section 1.2.1). For some proteins, the addition of denaturants results in conformations that retain some native-like secondary structure but exhibit an overall disrupted tertiary structure [13-18]. As a consequence, hydrophobic areas that were originally buried within the native protein structure are now exposed. Bis-ANS, a hydrophobic probe, is sensitive to the exposed hydrophobicity of the protein surface during unfolding and will bind to molten globule intermediates present [20, 22].

From time resolved emission anisotropy studies of PM-BPAO (Sections 3.2.2.2.5), the BPAO dimer is ultimately dissociated into monomers with the addition of GuHCl. Figure 55 shows the binding of 100-fold excess bis-ANS to BPAO unfolded by GuHCl. The fluorescence intensity of bis-ANS shows a shoulder at 2M (MG-1) and a peak in 3M GuHCl (MG-2). Bis-ANS intensity is 5 and 8 times greater than the intensity of the probe in native BPAO in 2 and 3M GuHCl respectively. There is a dramatic 8-fold decrease in intensity as GuHCl concentration is increased to 4M. At 5M GuHCl, the binding of bis-ANS is negligible which suggests that there are no hydrophobic pockets to which bis-ANS can bind. The data suggests the presence of possibly two molten globule intermediates (MG-1 and MG-2) during the denaturation of BPAO by GuHCl.

The first molten globule intermediate, MG-1, corresponds to the midpoint of the transition observed from tryptophan CSM experiments (Figure 29), whereas the GuHCl concentration associated with the second bis-ANS intermediate, MG-2, corresponds to the dissociation of the BPAO dimer from time resolved lifetime (Figure 45) and emission anisotropy (Figure 48) studies, as well as steady state anisotropy studies of tryptophan (Figure 34) and pyrene maleimide (Figure 41) (Table 16). It is also of interest to note that the intensity of bis-ANS in the refolded conformation (0.5M GuHCl) is comparable to that of the 3M GuHCl unfolded protein (Figure 55) rather than the native protein. Thus, the refolded protein conformation represents an alternate conformation as previously suggested from the tryptophan and pyrene maleimide total fluorescence intensities (Figure 34, *inset* and Figure 41, *inset*).

Figure 57 shows the result of titrating bis-ANS into BPAO perturbed to various extents by GuHCl. At low GuHCl concentrations (native, 0.5M unfolded and refolded), the binding of bis-ANS to the protein displays hyperbolic character (Equation 68) while at concentrations over 2M, the binding changes to a sigmoidal transition (Equation 67). This suggests a change to cooperative binding of bis-ANS to BPAO.

Similar molten globule intermediate studies were performed using urea as the perturbant. In contrast to GuHCl data (Figure 55), there was little sensitivity to the binding of 100- and 1000-fold excess bis-ANS to urea denatured BPAO (Figure 56). At two concentrations of fluorophore (2.5×10^{-5} and 1.0×10^{-4} M), there was no appreciable increase in bis-ANS binding. Therefore, urea does not appear to perturb the BPAO conformation through a molten globule intermediate. Further analysis by titration of various urea induced BPAO conformations with bis-ANS (Figure 58) shows the

influence of urea on bis-ANS fluorescence emission intensity. A key feature is the hyperbolic nature of the plots at most of the urea concentrations studied (except 8M which is sigmoidal in character (Equation 67)). This is similar to the effect at low GuHCl concentrations (0-0.5M; Figure 57) but the intensities are much lower with urea even though higher concentrations of bis-ANS ($1.4 \times 10^{-4}\text{M}$) were titrated.

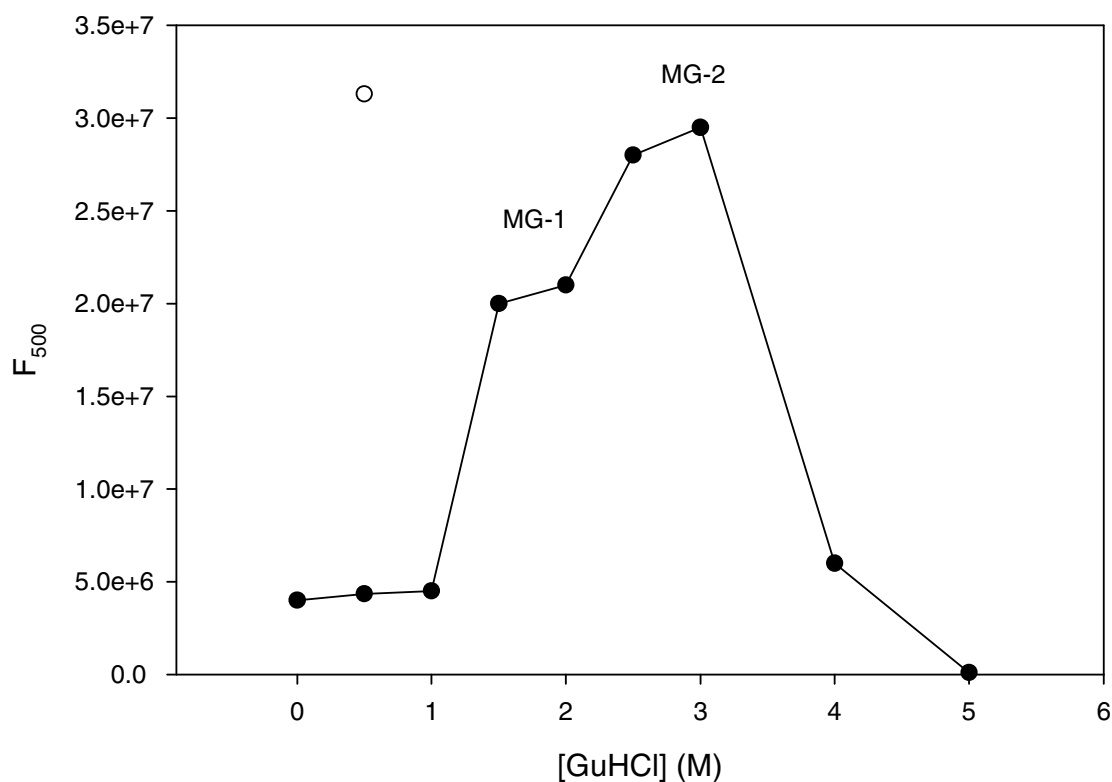


Figure 55: Effect of GuHCl on the emission intensity of bis-ANS. The concentration of BPAO in the unfolded (●) and refolded (○) solutions is 2.5×10^{-7} M. The bis-ANS concentration is 2.5×10^{-5} M. MG-1 and MG-2 are the different molten globule intermediate conformations. Bis-ANS is excited at 395nm and emission is monitored at 500nm. The excitation and emission path lengths are each 3mm. The line shows the trend.

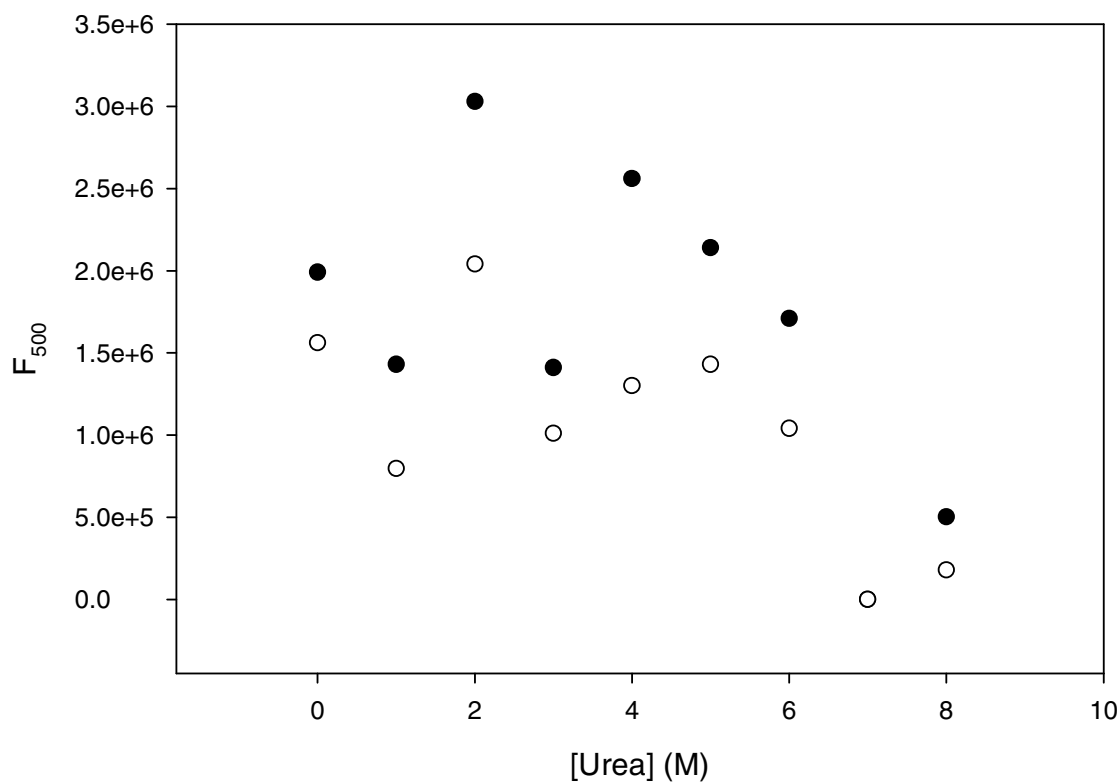


Figure 56: Effect of urea on the emission intensity of bis-ANS. The concentration of BPAO is 2.5×10^{-7} M and the concentrations of bis-ANS are 2.5×10^{-5} M (○) and 1×10^{-4} M (●). Bis-ANS is excited at 395nm and emission is monitored at 500nm. The excitation and emission path lengths are each 3mm.

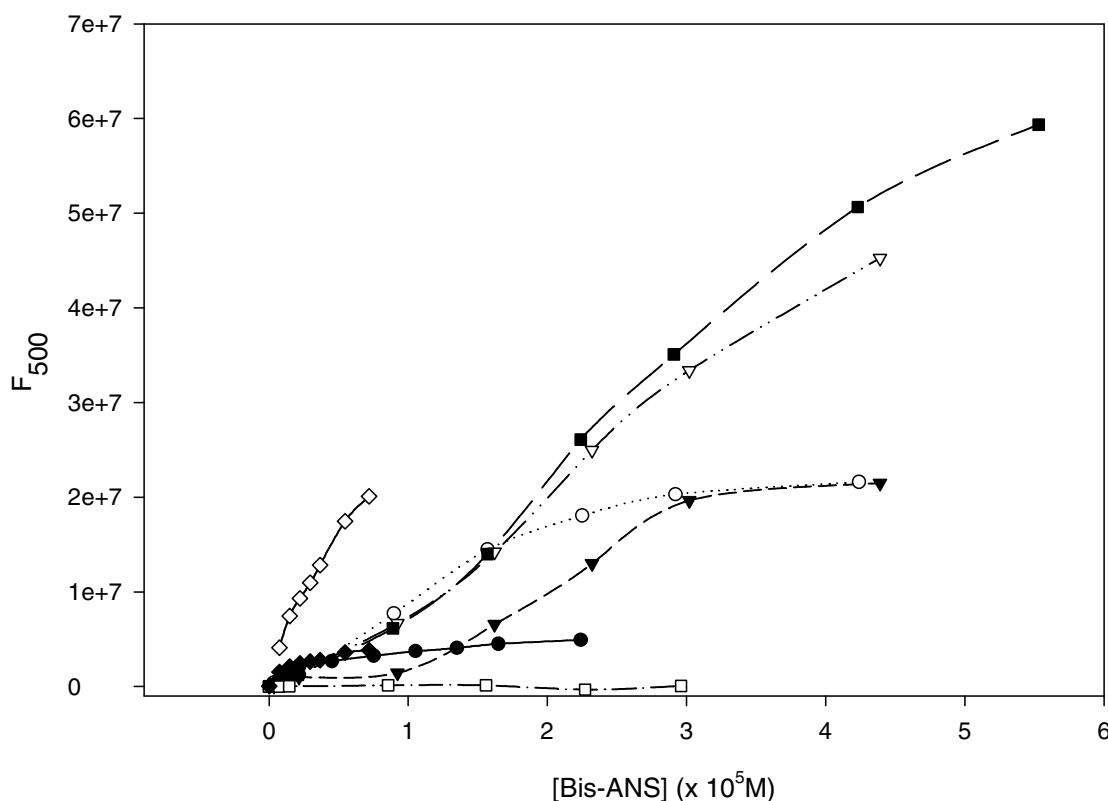


Figure 57: Titration of GuHCl perturbed BPAO conformations with bis-ANS. The concentration of BPAO is 2.5×10^{-7} M. The unfolding [GuHCl] are 0M ($\bullet$), 0.5M ($\blacklozenge$), 1.5M ($\circ$), 2M ($\blacktriangledown$), 2.5M ($\triangledown$), 3M ($\blacksquare$), and 5M ($\square$) and refolding is at 0.5M ($\diamond$). The denaturation lines represent the fit to either hyperbolic (0 – 1.5M; Equation 68) or sigmoidal functions (2 – 5M; Equation 67) and the refolding line show a hyperbolic characteristic (Equation 68). Bis-ANS is excited at 395nm and emission is monitored at 500nm. The excitation and emission path lengths are each 3mm.

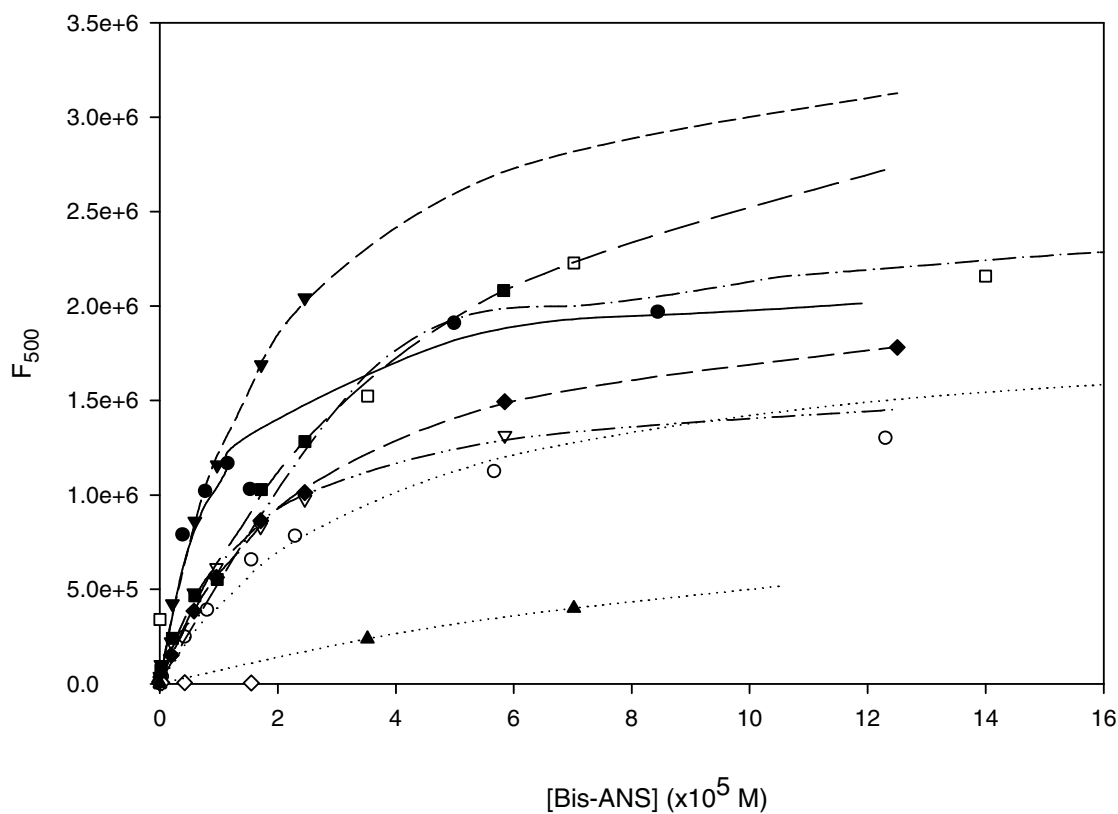


Figure 58: Titration of urea perturbed BPAO conformations with bis-ANS. The concentration BPAO is $2.5 \times 10^{-7} M$ and the concentration of urea is 0 (●), 1 (○), 2 (▼), 3 (▽), 4 (■), 5 (□), 6 (◆), 7 (◇) and 8M (▲). The lines represent hyperbolic fits to the data (Equation 68). Bis-ANS is excited at 395nm and emission is monitored at 500nm. The excitation and emission path lengths are each 3mm.

3.4 Local Environments

3.4.1 Active Site

Valuable information about altered conformational states of BPAO can be determined from changes in the specific activity of the protein with the addition of denaturants: GuHCl; urea; and SDS. Specifically, the integrity of the active site can be studied.

The effect of denaturant on the activity of BPAO was recorded as the residual specific activity (SA/SA_0) of the protein, using 0.02M benzylamine as the substrate. For GuHCl (Figure 59), a sharp decrease in residual enzyme activity was observed at low concentrations of GuHCl (0.02M), with a transition midpoint occurring at 3.5mM. The protein completely lost enzyme activity at 0.015M GuHCl, which is well below where conformational changes were observed from studies of tryptophan CSM, HPLC elution volumes, and pyrene maleimide emission anisotropy and rotational correlation times (Table 16).

A similar effect on BPAO activity was observed for urea (Figure 60). Increasing the concentration of urea to 3M resulted in a sigmoidal decrease in the residual activity with a transition midpoint at 1.30M. However, in general, higher concentrations of urea were required to achieve the same level of reduced activity observed with GuHCl. At urea concentrations greater than 3M, the residual activity of BPAO is negligible. Interestingly at low urea concentrations ($<0.1M$), an increase the activity by as much as 20% was noted.

For SDS, the decrease in residual activity of BPAO (Figure 61) follows spectroscopic (Figure 32) and electrophoretic (Figure 27) analyses with a transition

midpoint of the sigmoidal decrease in activity at ~1mM SDS. It is clear that a conformational effect occurs around the cmc of SDS, with dissociation of BPAO and the turnover of the benzylamine substrate to benzaldehyde is stopped.

It is important to note that GuHCl and urea (Figure 1) can both serve as competitive inhibitors of BPAO since they also have amine groups. Thus, it was unclear whether the decrease in specific residual activity for BPAO is the result of a conformational change in the protein structure, or caused by a competitive inhibition of BPAO by GuHCl and urea. Thus, the influence of inhibition of BPAO by GuHCl or urea was probed through inhibition experiments (Section 3.4.2).

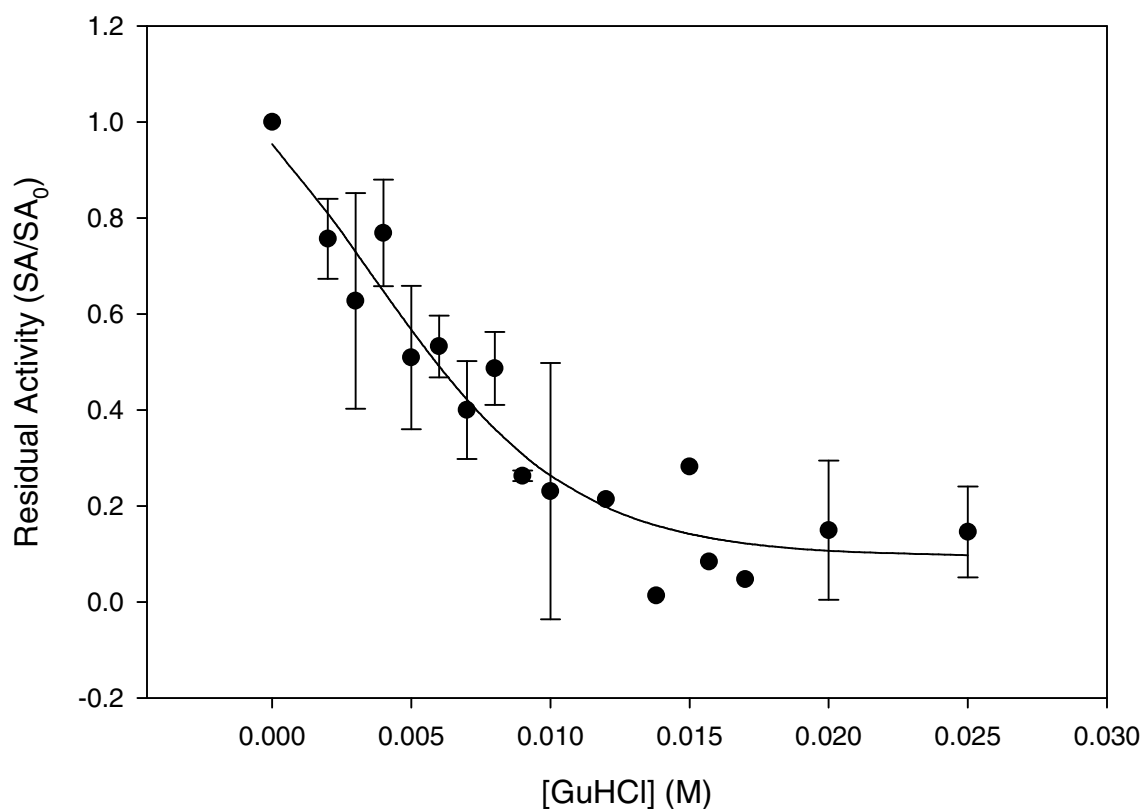


Figure 59: Effect of GuHCl denaturant on the residual specific activity of BPAO. The substrate is benzylamine (0.02M) and the concentration of BPAO is 2.5×10^{-7} M. Standard deviations reflect 4 trials. The line is the sigmoidal fit (Equation 67) to the data ($r^2=0.9171$) with a transition midpoint at 3.5mM GuHCl.

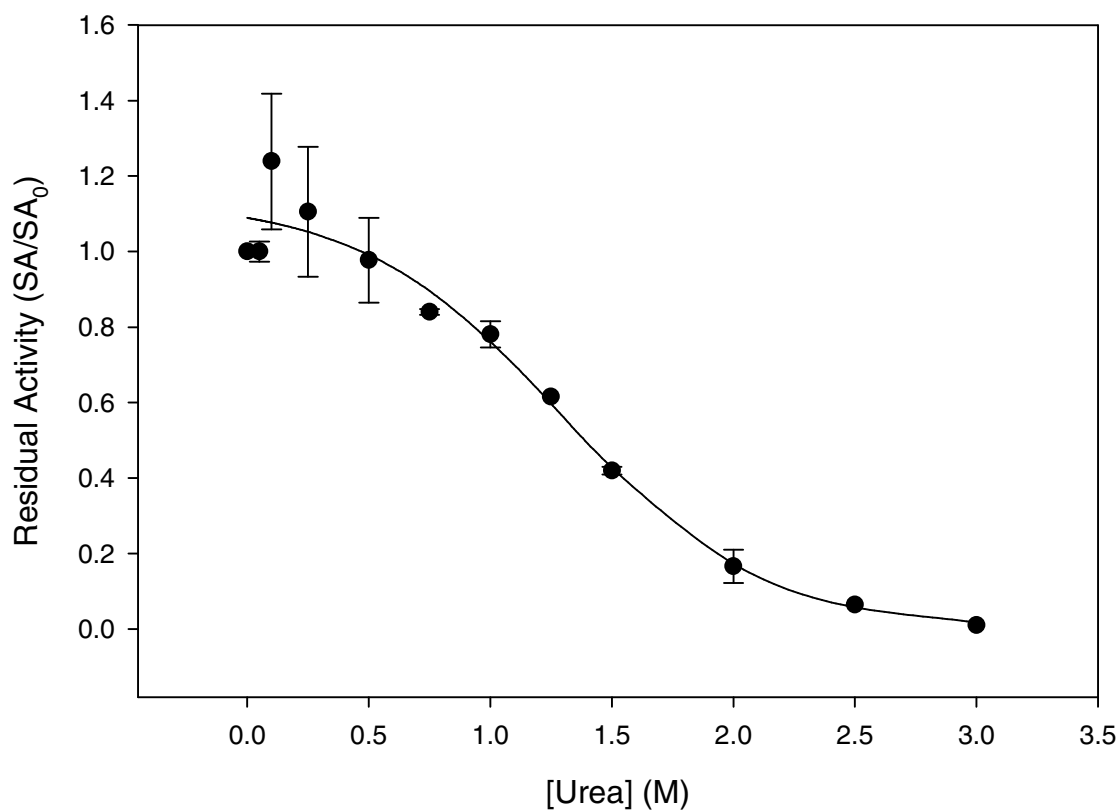


Figure 60: Effect of urea denaturant on the residual specific activity of BPAO. BPAO concentration is a constant $2.5 \times 10^{-7}\text{M}$ and the concentration of substrate (benzylamine) is 0.02M. The data represents the standard deviations for at least 2 trials. The line is the sigmoidal fit (Equation 67) to the data ($r^2 = 0.9757$) with a transition midpoint at 1.29M urea.

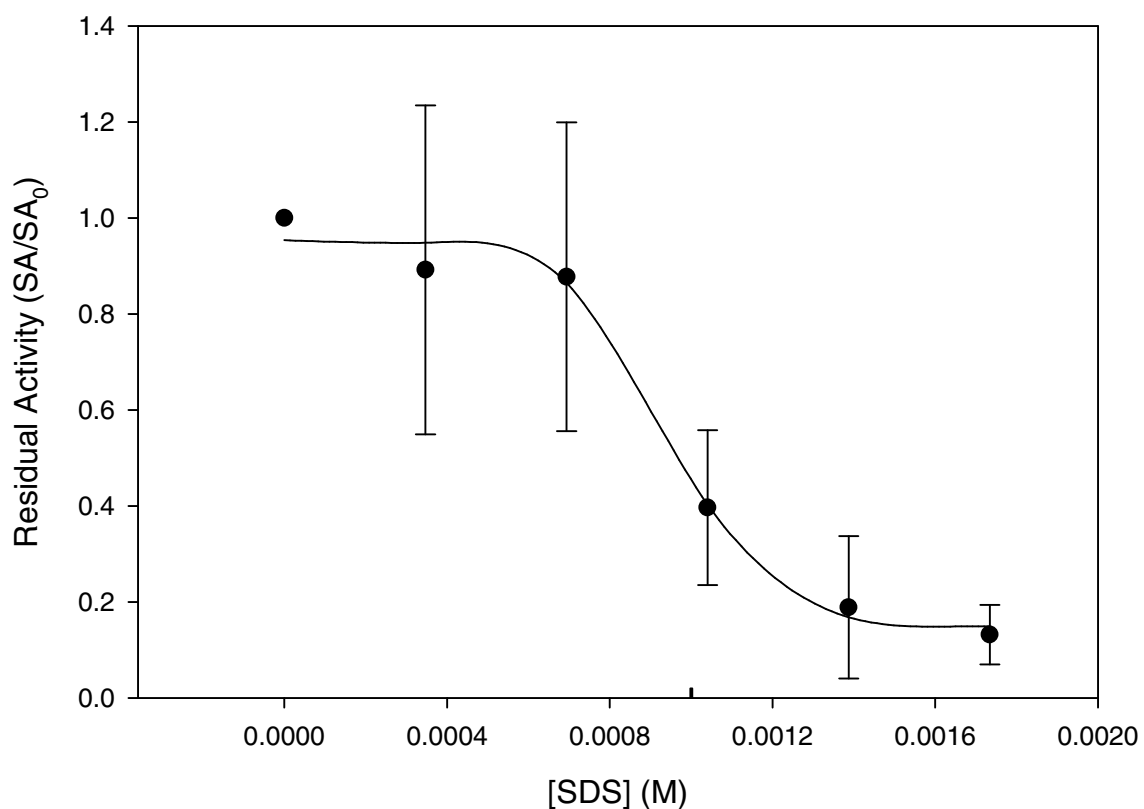


Figure 61: Effect of SDS denaturant on the residual specific activity of BPAO. The data represents the standard deviations of 4 trials. The line is the sigmoidal fit (Equation 67) to the data ($r^2 = 0.9693$) with a transition midpoint at 0.947mM SDS. The BPAO and benzylamine concentrations are 2.5×10^{-7} M and 0.02M, respectively. The cmc in 0.12M ionic strength is shown by the vertical line at 1mM SDS [29] for reference.

3.4.2 Inhibition of BPAO *versus* Conformational Change

In an attempt to elucidate whether a conformational change in BPAO or inhibition of the protein by the amine denaturant leads to a decrease in enzyme activity, enzyme inhibition studies were performed. Increasing concentrations of GuHCl (Figure 62) or urea (Figure 63) resulted in a decrease in velocity from the Michaelis-Menton plots (Equation 39). The kinetic data were then further analyzed using a Lineweaver-Burk double reciprocal plot (Equation 40) for GuHCl (Figure 62, *inset*) and urea (Figure 63, *inset*). The $V_{\max}$ for the protein (0.00554s^{-1}) was unaffected by both denaturants, however, K_m values increased. It is clear, therefore, that GuHCl and urea both exhibit competitive inhibition characteristics.

From the slopes of the double reciprocal Lineweaver-Burk plots of $(K_m/V_{\max})$ *versus* inhibitor concentration (Equation 42 and 43), the inhibition constants (K_i) for GuHCl and urea were estimated for the assay conditions. With GuHCl, there is a linear increase in the slope with concentration of denaturant (Figure 64). The inhibition constant, K_i , for GuHCl was determined to be 2.30mM for the 85% decrease in residual specific activity (Figure 59). However, for urea, a two phase plot was observed. For the first phase, urea concentrations up to 1M (Figure 65, *inset*) resulted in linear slopes for $K_m/V_{\max}$ *versus* $[I]$ and the K_i was determined to be 1.21M. This reflects a 20% decrease in residual specific activity (Figure 60) as a result of competitive inhibition. At urea concentrations greater than 1.0M (Figure 65), a dramatic exponential increase in the slope of $K_m/V_{\max}$ was observed which suggests more complex changes in the protein conformation and/or another mechanism involved.

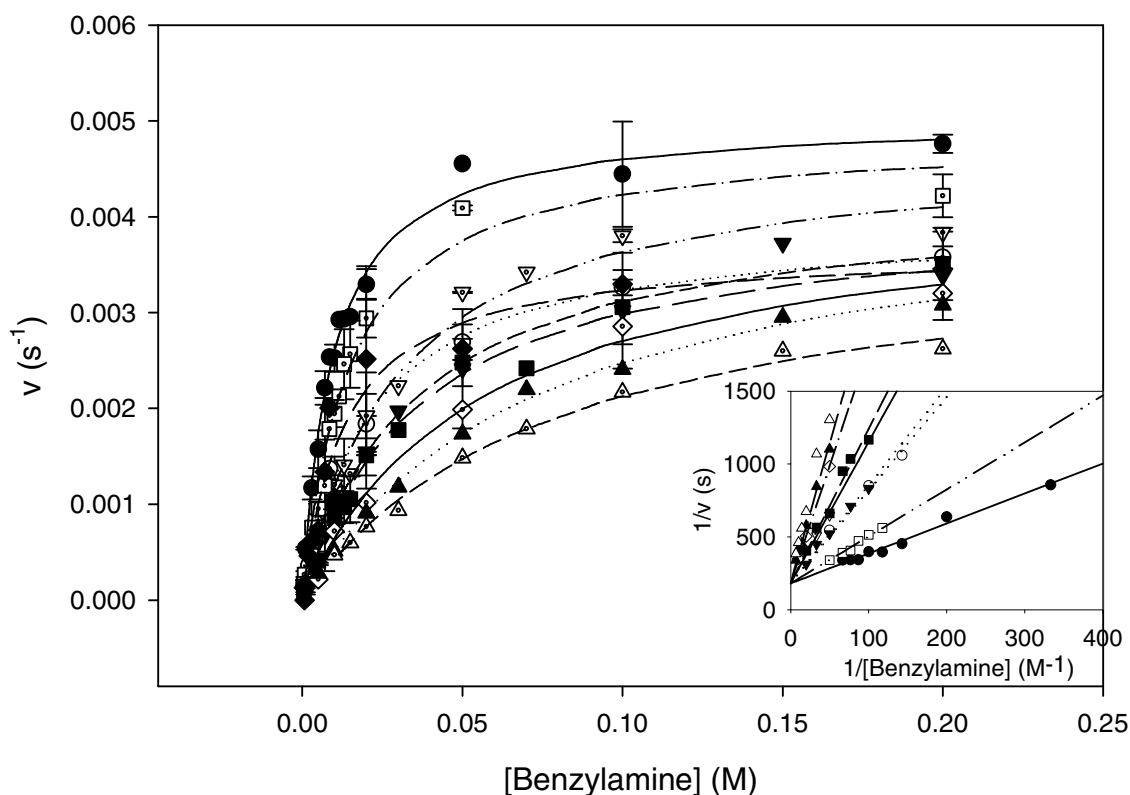


Figure 62: Effect of different concentrations of GuHCl on the velocity of BPAO. The lines represent the hyperbolic fit to the data (Equation 68). The standard deviations are representative of at least 2 trials. The GuHCl concentrations are 0 (●), 0.002 (□), 0.003 (◆), 0.005 (○), 0.006 (▽), 0.007 (▼), 0.01 (■), 0.015 (◇), 0.02 (▲) and 0.025M (△). [BPAO] is $2.5 \times 10^{-7}\text{M}$. The r^2 values are above 0.9600 except for 0.007M GuHCl, where it is 0.9316. The standard deviations are representative of at least 2 trials. *Inset:* The corresponding Lineweaver-Burk double reciprocal plot for GuHCl denatured BPAO. V_{max} is $5.54 \times 10^{-3}\text{s}^{-1}$. The lines are a linear fit of the data.

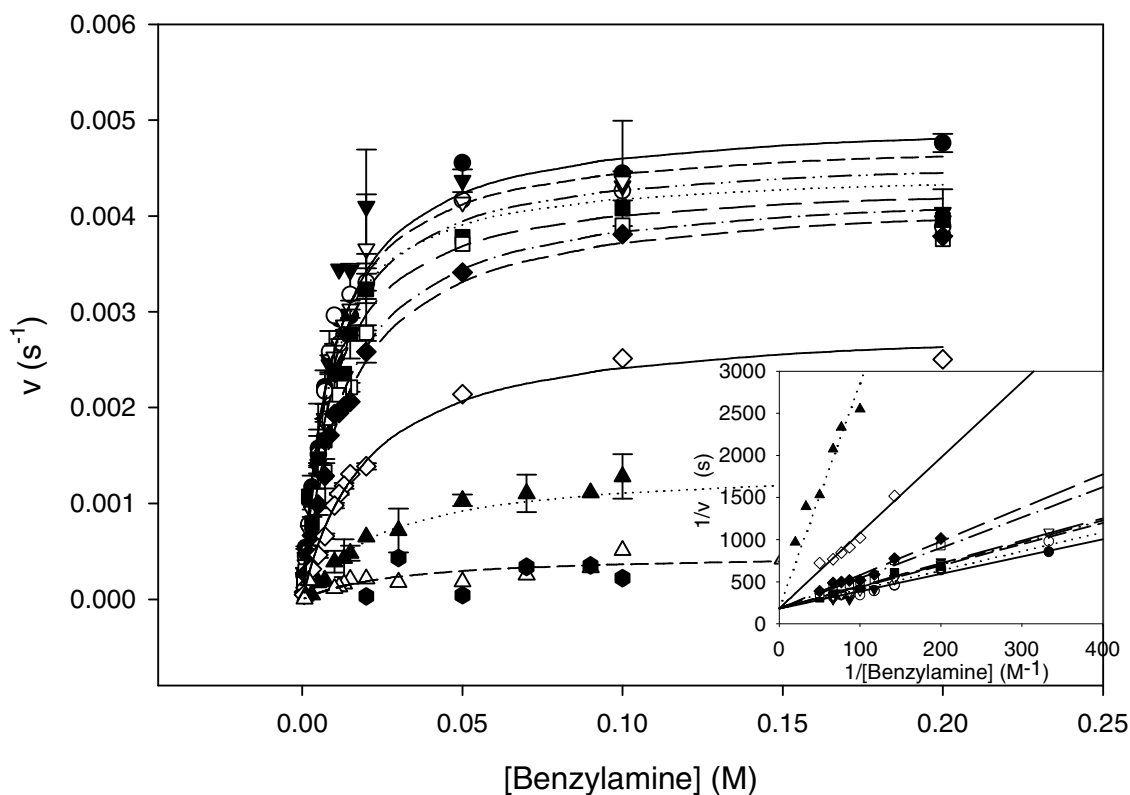


Figure 63: Effect of different concentrations of urea on the velocity of BPAO. The urea concentrations are 0M ($\bullet$), 0.05M ($\circ$), 0.1M ($\blacktriangledown$), 0.25M ($\triangledown$), 0.5M ($\blacksquare$), 0.75M ($\square$), 1.0M ($\blacklozenge$), 1.5M ($\diamond$), 2.0M ($\blacktriangle$), 2.5M ($\triangle$) and 3.0M ($\bullet$). The lines represent the hyperbolic fit to the data. The standard deviations are representative of at least 2 trials. $[\text{BPAO}]$ is $2.5 \times 10^{-7}\text{M}$. *Inset:* The resulting Lineweaver-Burk double reciprocal plot for urea denatured BPAO. The lines represent the linear fit of the data. V_{max} is $5.54 \times 10^{-3}\text{s}^{-1}$.

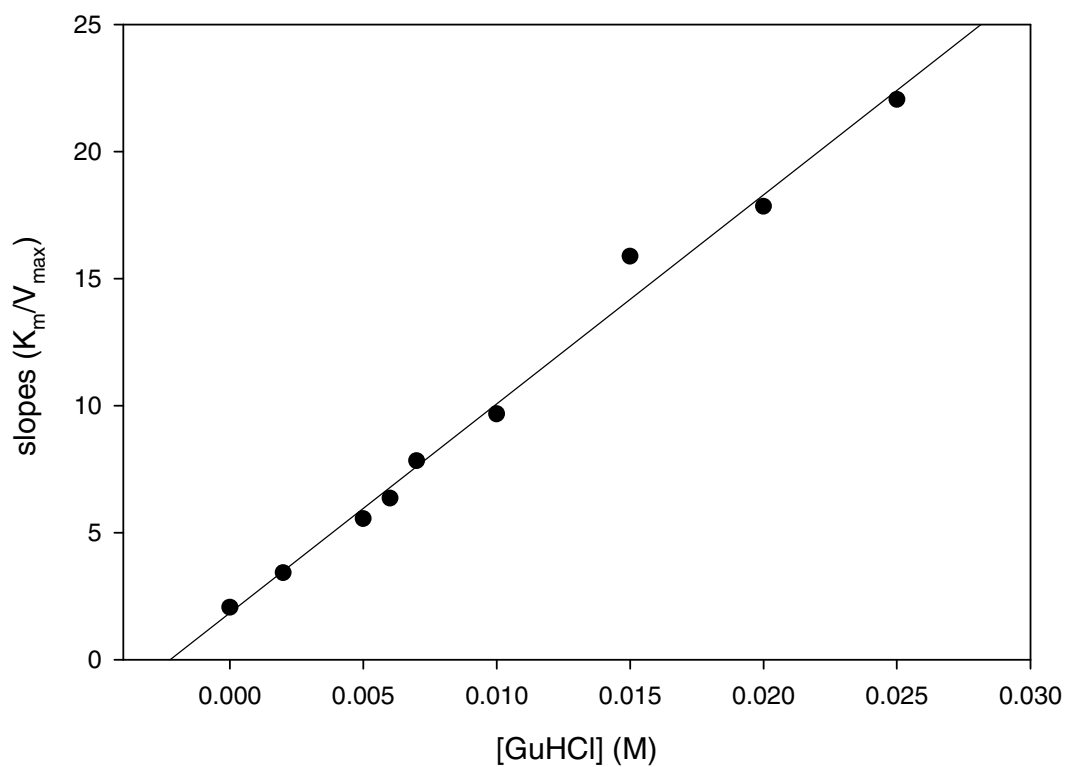


Figure 64: Analysis of the slopes of the Lineweaver-Burk double reciprocal plot in Figure 62 as a function of GuHCl denaturant concentration. The line is a linear fit of the data with a correlation coefficient, r^2 , of 0.9901 and a calculated K_i of 2.30mM GuHCl (Equation 43).

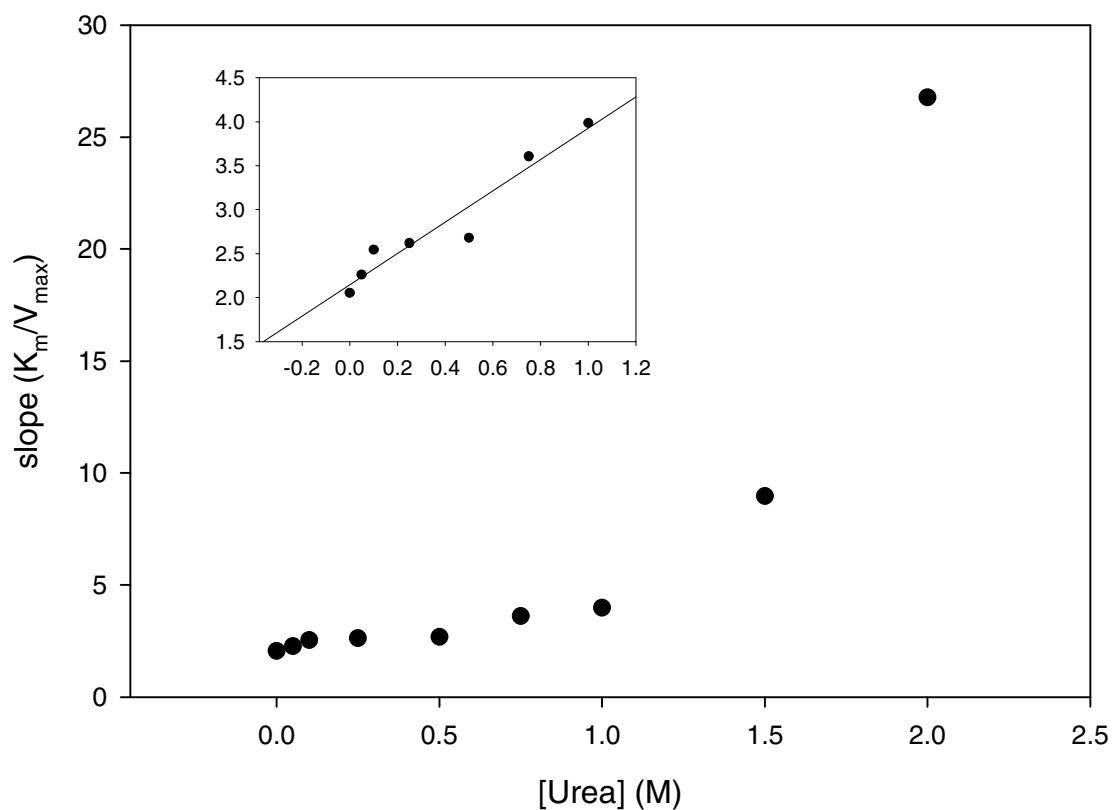


Figure 65: Analysis of the slopes of the Lineweaver-Burk double reciprocal plot in Figure 63 as a function of urea denaturant concentration. *Inset*: the slopes as a function of urea with a maximum concentration of 1M. The data is fit as a line with a correlation coefficient, r^2 , of 0.9319 and the calculated K_i is 1.21M urea (Equation 42 and 43).

3.5 Discussion

3.5.1 Proposed Pathways for the Interaction of Denaturants with BPAO

The ultimate aim of this research is to elucidate the denaturation pathways for BPAO in response to various chemical denaturants. Many experiments were conducted using a variety of biophysical techniques in order to further our understanding of the key components of the denaturation pathway for this large molecular weight homodimeric protein.

The Native Protein

The properties of the native dimeric BPAO conformation were investigated from protein fluorometric parameters (CSM, lifetime, steady state emission anisotropy, TREAA), hydrodynamic size (HPLC) and the hydrophobicity of the dimeric surface (bis-ANS and ANS binding).

The dimensions of the molecule during unfolding were probed by PM steady state and time resolved anisotropy (Sections 3.2.2.2.3 and 3.2.2.2.5), tryptophan anisotropy (Section 3.2.2.1.2), rotational correlation time (Section 3.2.2.2.5) and Stokes radius (Section 3.2.3). Pyrene maleimide anisotropy measures the rotations of the entire macromolecule as well as domain unfolding (Section 1.5.1.1.2.1). The steady state emission anisotropy value determined from time resolved parameters, $\langle r \rangle_{TR}$, is 0.153 (Figure 48) and an average steady state emission anisotropy, $\langle r \rangle_{SS}$, of 0.146 (Section 3.2.2.2.3). PM-BPAO exhibits three rotational correlation times: 164, 9 and 0.39ns for ϕ_{long} , ϕ_{medium} and ϕ_{short} (Table 13), which may be assigned to rotational motions of the intact dimer, local domain rotations and fast rotations of the fluorophore itself,

respectively. The corresponding radius for the dimer from ϕ_{long} is 56.6 Å (Section 3.2.2.2.5). As expected, the fractional contribution of the dimer ($\beta_{\text{long}} \phi_{\text{long}}$) is the predominant species in solution (99%; Figure 47). The HPLC elution volume corresponds to a protein molecular weight of 192kD (Figure 49, Table 15) with a Stokes radius of 55.4Å (Section 3.2.3). Table 15 provides a summary of the molecular weights determined from the various methods, which is about 200kD.

The surface of native BPAO has exposed hydrophobic patches to which ANS and bis-ANS binds relatively tightly with dissociation constants of $1.33 \pm 0.27 \times 10^{-5} \text{M}$ (Figure 25) and $7.9 \times 10^{-6} \text{M}$ (Figure 23), respectively.

From spectroscopic, chromatographic and enzymatic analyses, the effects of SDS, urea and GuHCl on the folding and denaturation pathways of BPAO appear to be quite different. In general, the experimental parameters were fit using a two-state model of the denaturant concentration at the transition midpoint (Equation 67). Table 16 represents a summary of the results for all the methods investigated.

Table 15:
Experimentally determined molecular weight of BPAO using different methods.

Technique	Molecular Weight (D)
Amino Acid Residues	169495
HPLC	192000
SDS-PAGE	194000

3.5.1.1 SDS

SDS is an anionic amphiphilic molecule that consists of a hydrophobic tail and hydrophilic head region (Figure 1). In 0.12M ionic strength buffer, the critical micelle concentration (cmc) is ~1mM SDS [29]. Figure 66 and Equation 74 summarizes the results of SDS on BPAO. The first transition, assigned as the dimer to monomer dissociation process, occurs at 0.91 ± 0.04 mM SDS and may reflect the formation and interaction of micelles with the protein (i.e. the cmc). It is monitored by SDS-PAGE (Figure 27), residual specific activity (Figure 61) and pyrene maleimide steady state emission anisotropy (Figure 43). Dissociation of the dimeric BPAO conformation leads to the decrease in enzyme activity. Unfolding of the monomer (M^U) was suggested from tryptophan integrated intensity data, with a mid-point at 3.1mM SDS (Figure 32). Monomeric unfolding occurs at approximately 3 times higher SDS concentration than necessary for dissociation. The dissociative and monomeric unfolding denaturation effect of SDS on BPAO conformation may be summarized by:



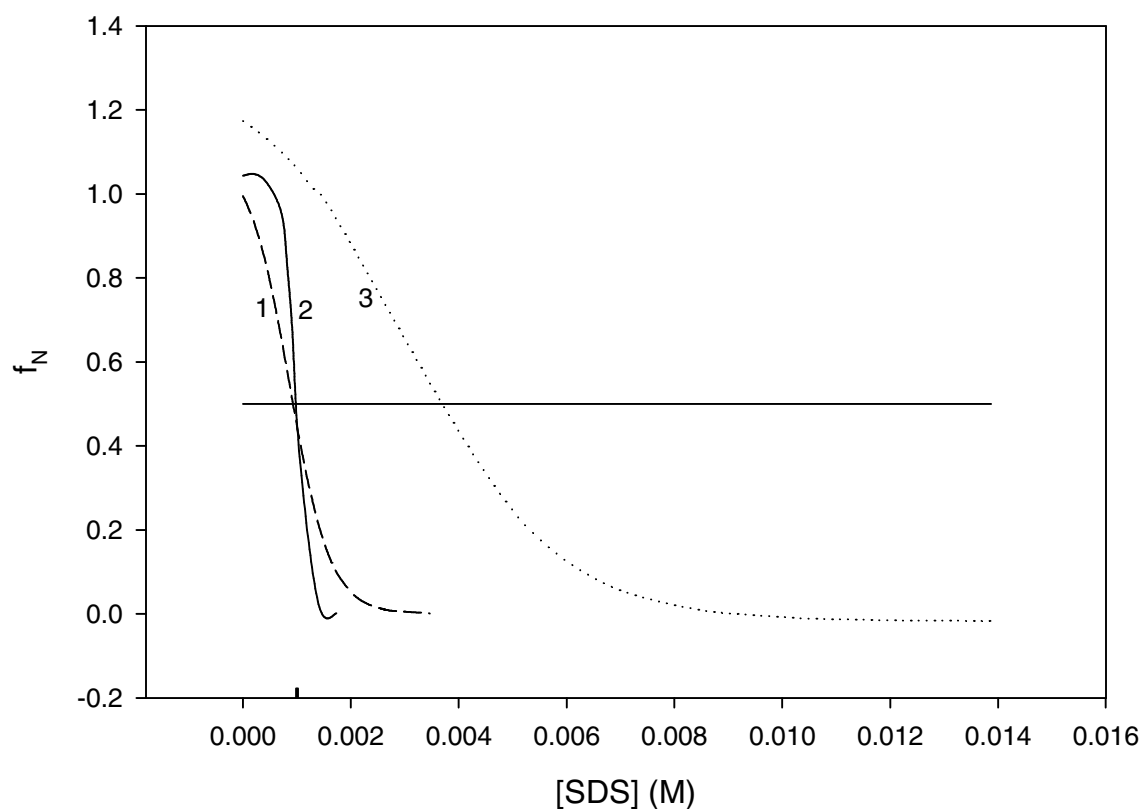
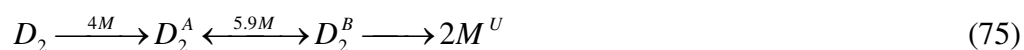


Figure 66: SDS-induced denaturation of BPAO by various techniques: pyrene maleimide anisotropy (1), residual activity (2) and normalized integrated intensity from tryptophan fluorescence (3). The lines are the sigmoidal fit to the data (Equation 67) and a line is shown at $f_N = 0.5$ for clarity. f_N is calculated by Equation 69. The cmc in 0.12M ionic strength is shown by the vertical line at 1mM SDS [29].

3.5.1.2 Urea

The effect of neutral urea on BPAO can be summarized by Equation 75 and Figure 67. At low concentrations (<1M), urea is a competitive inhibitor for BPAO ($K_i=1.21\text{M}$, Figure 65). The first conformational transition for the protein (4.03M) in urea (D_2^A) was monitored by tryptophan anisotropy (Figure 35). The next transition midpoint (5.93M) is determined from CSM studies (Figure 30), which also reveal hysteresis upon renaturation. Between 5 - 6M urea, UGGE (Figure 26) shows the formation of an intermediate in equilibrium with the unfolded BPAO dimer and after 6M, the predominant form is the unfolded dimeric conformation (D_2^B).

Pyrene maleimide anisotropy data (Figure 42) suggests some partial dissociation for BPAO, but the transition to the monomer is incomplete even in 8M urea and the protein is essentially an unfolded dimer. A similar incomplete transition is seen from the increase in Stokes radius (Figure 54). HPLC does not show the presence of a folded monomeric peak (Section 3.2.3). Therefore, the dimeric BPAO intermediate (D_2^B) does not dissociate into a folded monomer (M) but an unfolded monomer (M^U). Furthermore, there is no molten globule intermediate conformation with urea denaturation (Figure 56). The unfolding and dissociative effect of urea on the BPAO dimer may be represented as:



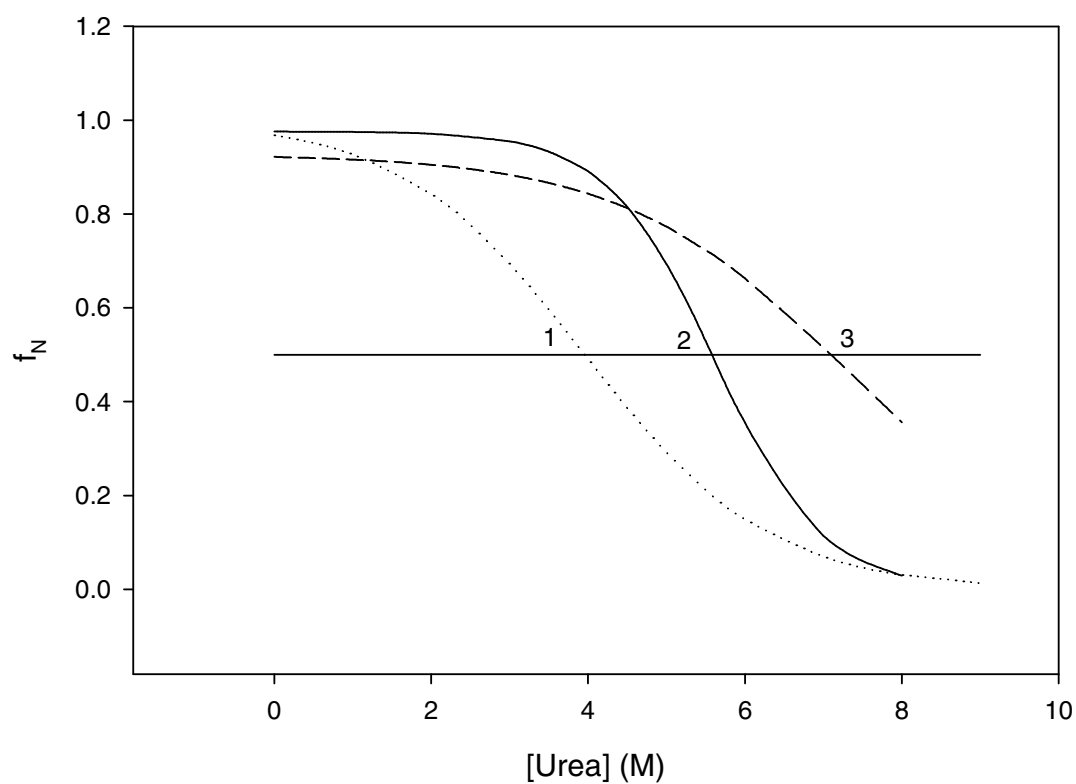
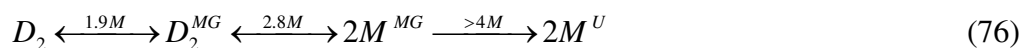


Figure 67: The fraction of native BPAO dimer present upon urea-induced denaturation of BPAO by various techniques: steady state tryptophan anisotropy (1), tryptophan CSM (2) and pyrene maleimide anisotropy (3). The lines are the sigmoidal fits to the data (Equation 67) and a line is shown at $f_N = 0.5$ for clarity. f_N is calculated by Equation 69.

3.5.1.3 GuHCl

In general, the effect of GuHCl on BPAO may be summarized in Figure 68 and Equation 76. GuHCl competitively inhibits BPAO ($K_i = 2.30\text{mM}$; Figure 64) at low concentrations ($<0.025\text{M}$; Figure 59 and 63). The first GuHCl conformational transition is a reversible change in tryptophan CSM (Figure 29) and tryptophan total intensity (Figure 34, *inset*) to an unfolded dimer that has some molten globule characteristics (MG-1; Figure 55), D_2^{MG} , at $1.9 \pm 0.3\text{M}$ GuHCl. This is followed by the reversible dissociation of the molten globule dimer into a molten globule monomer (MG-2; Figure 55), M^{MG} at $2.8 \pm 0.3\text{M}$ GuHCl as monitored by tryptophan anisotropy (Figure 34), τ_{long} and τ_{short} (Figure 45), the fractional rotational correlation times of ϕ_{long} and ϕ_{medium} (Figure 47) and Stokes radii (Figure 52) transition midpoints (Table 16). At concentrations greater than 4M GuHCl, the monomer unfolds (M^U) as evidenced by the lack of bis-ANS binding (Figure 55). Thus, the unfolding and dissociation of BPAO by GuHCl may be represented by:



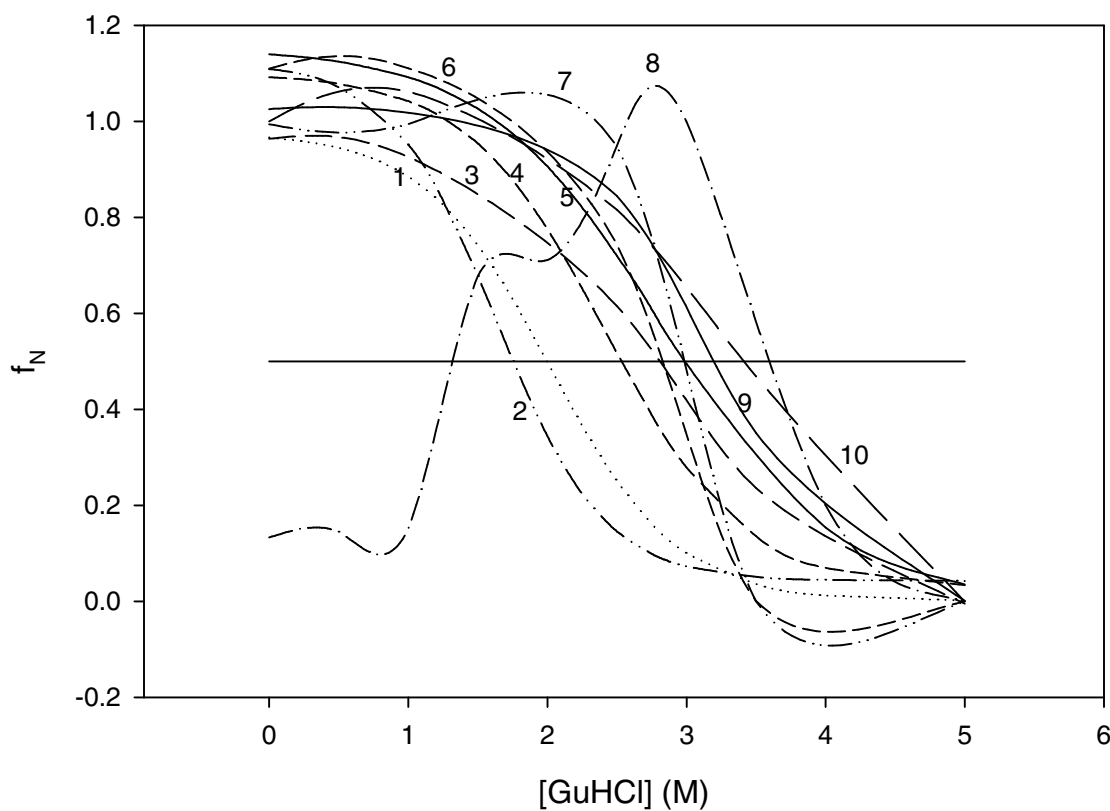


Figure 68: GuHCl-induced denaturation of BPAO by various techniques: tryptophan CSM (1), total intensity from tryptophan anisotropy (2), τ_{long} (3), tryptophan anisotropy (4), Stokes Radii (5), ϕ_{long} (6), ϕ_{medium} (7), Bis-ANS fluorescence intensity (8), τ_{medium} (9) and time resolved pyrene maleimide anisotropy (10). The line at $f_N = 0.5$ is shown for clarity. f_N is calculated by Equation 69. The bis-ANS data is normalized to 0M and the peak. All lines represent the sigmoidal trend (Equation 67), except 10 which shows the linear trend.

Table 16:
Summary of transition midpoints for denaturants by various experimental
methods used in these studies.^a

Technique	GuHCl (M)		Urea (M)		SDS (mM)
	denatured	renatured	denatured	renatured	denatured
Residual Activity	0.0035		1.29		0.95
Tryptophan CSM	2.10	1.80	5.93	4.79	6.67 ^b
Trp II					3.1
Elution Volume	2.23	2.25			
Stokes radii	2.74	2.62			
$\langle r \rangle_{PM}$	2.45	2.63			0.94
$\langle r \rangle_{PM}$ Total Intensity					0.17 ^b
$\langle r \rangle_{trp}$	2.41	1.95	4.03		
$\langle r \rangle_{trp}$ Total Intensity	1.67				
$\langle r \rangle_{TR}$	3				
τ_{long}	2.88				
τ_{medium}	3.22				
$f_{\beta\phi long}$	2.75				
$f_{\beta\phi medium}$	2.69				

^a Midpoint transitions calculated from a sigmoidal fit (Equation 67) unless otherwise noted.

^b This represents a hyperbolic fit (Equation 68) and the denaturant concentration at 50% change in y is shown.

3.5.2 Free Energies of Unfolding and Dissociation

Thermodynamics of denaturation ($\Delta G_{H_2O}^0$) can be calculated for reversible processes (Section 2.2.9). Based on the pathways suggested in Section 3.5.1 for SDS, urea and GuHCl, it is possible to calculate the free energy of denaturation in the absence of denaturant (Equation 4) assuming all processes are reversible and each transition reflects a two-state transition [64].

Table 17 summarizes the results for all denaturants according to the method used and the concentration of BPAO. The three denaturants interacted with the BPAO dimer and lead to the unfolding of the dimer, dissociation of the dimer into monomers and/or unfolding of the monomers subsequent to dissociation. The stability of the various unfolded dimeric conformational transitions (ΔG_U^D) of BPAO (D_2^A , D_2^B and D_2^{MG}) is between 1.76 to 2.99 kcal/mol in GuHCl and urea. The free energy for the dissociation (ΔG_D) of BPAO dimer to monomers (M) in SDS was calculated to be an average of 10.5 ± 0.07 kcal/mol (from residual activity and pyrene maleimide steady state anisotropy data) and dissociation of the molten globule dimer to a molten globule monomer in GuHCl (M^{MG}) is an average of 12.8 ± 0.8 kcal/mol (from pyrene maleimide time resolved anisotropy, tryptophan steady state anisotropy and Stokes radii studies) which are comparable (Table 17). Monomeric unfolding (M^U), from SDS studies, has the low free energy (ΔG_U^M) of 1.12 kcal/mol which is similar to that of dimeric unfolding but much less than for dissociation.

The $\Delta G_{H_2O}^0$ value is independent of the denaturant used. Rather, it is based on the intrinsic stability of the protein itself assuming the unfolded conformation is the same in

different denaturants. The total $\Delta G_{H_2O}^0$ (ΔG_{total}) is the sum of the individual contributions for the dimer and twice those of the monomer (Equation 72). They are shown in bold in Table 17 for each denaturant. It is clear that the ΔG_{total} of denaturation for the three denaturants studied are different. The ΔG_{total} in urea is 4.75kcal/mol while in SDS and GuHCl it is 12.7 and 15.3 kcal/mol respectively. The difference in ΔG_{total} between GuHCl (15.3 kcal/mol) and SDS (12.7 kcal/mol) is 2.6kcal/mol which is within experimental error. The ΔG_{total} value for urea (4.75 kcal/mol), which reflects only unfolding of the dimer and not dissociation, is considerably lower than that determined for SDS and GuHCl. Therefore, this suggests that the final state of BPAO in urea is different than in SDS and GuHCl. In summary, for urea, the denaturation of BPAO to the unfolded state is incomplete.

The m value is the change in $\Delta G_{H_2O}^0$ with respect to the change in denaturant concentration (Equation 13). It is related to the accessible surface area [69]. With SDS, dissociation represents 73% of the m_{total} (2356 ± 104 kcal.mol⁻¹.M⁻¹) while monomeric unfolding accounts for the remaining 27% (429 kcal.mol⁻¹.M⁻¹). In contrast, GuHCl induced dimeric unfolding represents 45% of the total m value (1.21 kcal.mol⁻¹.M⁻¹) while dissociation accounts for 55% (1.45 ± 0.23 kcal.mol⁻¹.M⁻¹). Therefore, the accessible surface area is dependent on the mechanism of BPAO denaturation.

The transition midpoint values (C_m) determined by Equations 5 and 9 using $\Delta G_{H_2O}^0$ and m values for unfolding and dissociation (Table 17), are in good agreement with the transition midpoints in Table 16 determined from the sigmoidal two-state transition (Equation 67).

Table 17:
Thermodynamics for the denaturation of BPAO assuming two-state transitions.^a

Denaturant	[BPAO] (x10 ⁷ M)	Method	Intermediate formed	$\Delta G_{H_2O}^0$ (Kcal/mol)	m (kcal/(mol. M))	Cm ^b
SDS	6.72	$\langle r \rangle_{PM}^{SS}$ ^c	M	10.5	2429	0.64
	2.5	SA ^c	M	10.4	2282	0.80
	4.57	norm II	M ^U	1.12	429	2.61
				12.7^d	3214^d	
Urea	2.5	$\langle r \rangle_{trp}$	D ₂ ^A	1.76	0.438	4.02
	1.00	CSM	D ₂ ^B	2.99	0.631	4.74
				4.75	1.07	
GuHCl	2.13	CSM	D ₂ ^{MG}	2.48	1.21	2.05
	8.25	$\langle r \rangle_{TR}$ ^c	M ^{MG}	12.1	1.19	3.18
	2.5	$\langle r \rangle_{trp}$ ^c	M ^{MG}	12.5	1.53	2.30
	2.5	R _s ^c	M ^{MG}	13.7	1.63	2.87
				15.3^d	2.66^d	

^a Thermodynamics determined as a two state transition for unfolding by Equation 10 with a Cm calculated by Equation 5, unless otherwise noted.

^b The concentration units for BPAO, GuHCl and Urea are M and SDS is expressed in mM.

^c Experimental data analyzed as dissociation according to Equation 12 with the Cm for dissociation calculated by Equation 9.

^d The average dissociation free energy (ΔG_D) is used to calculate the total free energy (Equation 72).

Bold indicates ΔG_{total} (Equation 72) and m_{total} (Equation 73) for the conformational changes in each denaturant.

Chapter 4: Conclusions and Further Studies

Unlike the folding of small molecular weight globular proteins, multidomain multisubunit proteins are not well characterized. Denaturation pathways, the associated thermodynamics and rules that govern such pathways will provide a better understanding of the nature of the forces that stabilize the protein, especially the contribution of the dimeric interface stability to the overall stability of the protein. The protein under investigation is bovine plasma amine oxidase (BPAO). It is a multidomain homodimeric protein. Limited conformational characterization has been previously performed (Section 1.4.1.7) and the pathways for denaturation are unknown.

The aim of this research was to probe the denaturation conformations of BPAO during unfolding. This was achieved with the use of three different denaturants: cationic GuHCl, anionic SDS and neutral urea (Figure 1). Each denaturant interacts with the protein and exhibits different mechanisms for denaturation (Section 1.3.1) and therefore, the pathways of denaturation are expected to be different. From our studies:

1. SDS dissociates the BPAO homodimer and then further unfolds the monomer (Figure 69).
2. Urea, even at concentrations of 8M, unfolds the dimer but only partially dissociates the BPAO dimer (Figure 70).
3. GuHCl unfolds the dimer followed by dissociation into unfolded monomers (Figure 71).

Small conformational change in a protein usually results in inactivation [50, 71, 75, 82, 91, 219, 220]. In SDS, urea and GuHCl, BPAO inactivation occurs first. However, this effect appears to arise from a dissociation conformational change for SDS (Section 3.4.1) and substrate inhibition for urea and GuHCl (Section 3.4.2).

With the exception of inactivation, other conformations present during the denaturation of a dimeric protein are unfolded dimer, monomer, unfolded monomer or aggregates. The presence of an unfolded dimeric intermediate conformation is clearly evident for urea (Figure 70) and GuHCl (Figure 71) denaturation, but not with SDS (Figure 69). Urea denaturation shows the presence of two unfolded dimeric conformations (D_2^A and D_2^B) neither of which exhibit molten globule characteristics (Section 3.5.1.2). In contrast, GuHCl has a molten globule dimeric conformation (D_2^{MG}) present during denaturation (Section 3.5.1.3).

In addition to an unfolded dimeric conformation, denaturation of a dimer can also proceed via dissociation, as detected for BPAO with SDS and GuHCl, or by unfolding of the monomer (M^U) which is seen or expected at high concentrations of SDS, urea and GuHCl. In SDS, BPAO inactivation is concurrent with dissociation. The mechanism of SDS denaturation involves the stabilization of hydrophobic interactions by the hydrophobic tails (Section 1.3.1). The active site in BPAO is composed of residues from each monomer (Figure 9a). As a result, small changes in the conformation of one monomer is communicated through to the active site of the other. In contrast, BPAO dissociation in GuHCl appears to occur from a molten globule dimer (D_2^{MG}) to a molten globule monomeric conformation (M^{MG}) (Section 3.5.1.3).

While the pathways of denaturation in the denaturants studied are quite different, the overall free energy of denaturation is expected to be the same as this parameter is an intrinsic characteristic of the protein and not the denaturant. The total free energy of denaturation (Equation 72) determined from the linear extrapolation from the denaturation of BPAO (Section 2.2.9) is between 12.7-15.3 kcal/mol for GuHCl and SDS, but only 4.75 kcal/mol with urea (Table 17; Section 3.5.2). The low free energy value calculated for urea denaturation of BPAO reflect an alternate (incomplete) final state for the protein, which we predict to be the unfolded dimer. Thus, we conclude that a great deal of free energy for BPAO is tied up in subunit association. This is expected from strong interfacial interactions.

Previous studies on BPAO have focused on defining the active site structure of BPAO to elucidate the role of the copper cofactor [106-111] but few studies thus far have focused on the conformational stability of the protein [138, 142, 161]. The information presented in this study provides a detailed view of the native BPAO conformation and other intermediate conformations present during denaturation.

Future studies on the molecular characterization will focus on verifying the assumed reversibility of denaturation. Further, it is important to determine the point where the metal cupric cofactor dissociates from BPAO and the free energy of stabilization associated with the metal. The presence of copper may stabilize or have no effect on the thermodynamics of BPAO. Other metalloproteins have been reported to show both effects [89, 266].

The conformational characterization of intermediates for BPAO presented here reflects not only possible denatured states for other copper amine oxidases but provides

information about changes in the structure of dimeric proteins with three denaturants. It is important to understand the nature of the interactions that stabilize oligomeric multidomain proteins. Since BPAO has multiple domains, it is an appropriate protein model to study the unfolding and dissociation pathways. This information will contribute to our understanding of the stabilities and denaturation of other large molecular weight proteins.

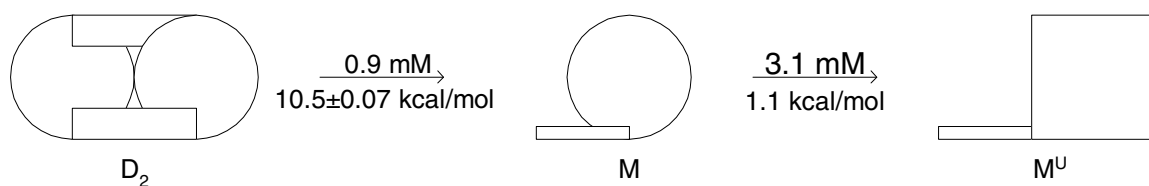


Figure 69: Dissociation and Unfolding Pathway for the Denaturation of BPAO by SDS. The midpoint transitions for each conformational change and the free energy of denaturation are shown above and under the arrow, respectively.

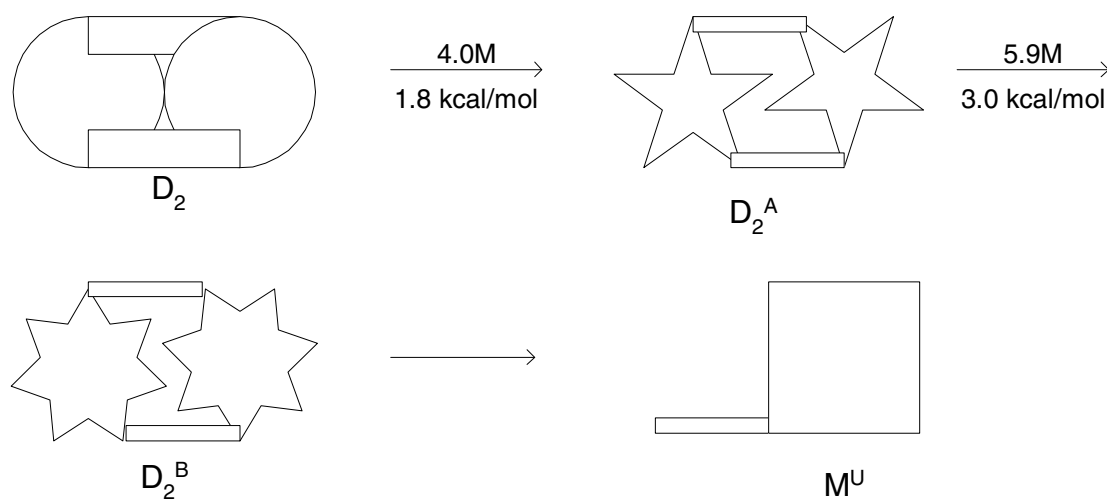


Figure 70: Dimeric Unfolding Pathway for the Denaturation of BPAO by Urea. The midpoint transitions for each conformational change and the free energy of denaturation are shown above and under the arrow, respectively.

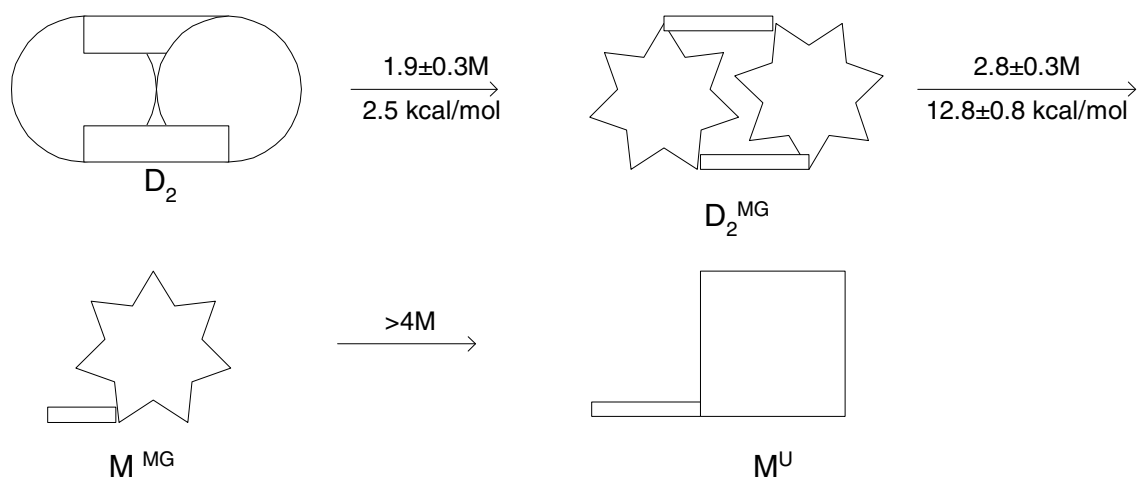


Figure 71: Unfolding and Dissociation Pathway for the Denaturation of BPAO by GuHCl. The midpoint transitions for each conformational change and the free energy of denaturation are shown above and under the arrow, respectively.

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