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PRERESONANCE RAMAN SPECTROSCOPIC STUDIES
ON RETINOID BINDING PROTEINS:
RBP, CRBP-I AND CRALBP

b y

ZHONGMO JU

A dissertation submitted to the Graduate Faculty in Physics
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, The City University of New York

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Abstract

PRERESONANCE RAMAN SPECTROSCOPIC STUDIES ON RETINOID BINDING PROTEINS: RBP, CRBP-I and CRALBP

b y

Zhongmo Ju

Advisor: Professor Robert H. Callender

The nature of the binding interactions between retinoid and many retinoid binding proteins is not well understood. Three such proteins: Retinol Binding Protein (RBP), Cellular Retinol Binding Protein-I (CRBP-I) and Cellular Retinaldehyde Binding Protein (CRALBP) have been isolated, complexed with all-*trans* and 11-*cis* retinal respectively, and probed using pre-resonance and difference Raman spectroscopy.

In the study of RBP, we have measured the pre-resonance Raman spectra of retinal, retinoic acid and retinol in dilute CCl₄ solutions and when bound to bovine-serum RBP. The comparison reveals that the binding interaction does not involve any specific interactions of the terminal group and the polyene chain with a particular protein residue. The data indicate hydrogen bonding of bound retinal's head

group oxygen to water, as well as some torsional angle change of its polyene chain upon binding.

The pre-resonance Raman spectrum of all-*trans* retinal complexed with CRBP-I has also been obtained, displaying a spectral shift in the position of the retinal carbonyl band that is typical of hydrogen bonding effects. This carbonyl red shift has been compared to hydrogen bonding interactions between the all-*trans* retinal carbonyl and a series of phenol derivatives, varying in proton donating ability by FT-IR spectroscopy, allowing quantitation of the hydrogen bond enthalpy.

To characterize the bonding between CRALBP and the carbonyl of 11-*cis* retinal, a difference Raman experiment was performed using protein complexes with native 11-*cis* retinal and 11-*cis* retinal isotopically labeled with ^{13}C at the 15 position. The difference spectrum has been compared to solution Raman spectra of 11-*cis* retinaldehyde, Schiff Base and Protonated Schiff Base in order to evaluate the retinoid CRALBP link. The results point to a possibility either Schiff Base or H-bond as the linkage between CRALBP and 11-*cis* retinal. Future isotopic labeling study of CRALBP complexed with ^{18}O -edited 11-*cis* retinal is designed to clarify this confusion.

This thesis is dedicated to my parents

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ABBREVIATIONS

RBP	Retinol Binding Protein
CRBP-I	Cellular Retinol Binding Protein Type I
CRBP-II	Cellular Retinol Binding Protein Type II
CRALBP	Cellular Retinaldehyde Binding Protein
TTR	Transthyretin
SB	Schiff Base -C=N-
PSB	Protonated Schiff Base -C=NH ⁺ -
HOOP	Hydrogen-Out-Of-Plane Bending
15- ¹³ C-retinal	retinal dehyde with ¹³ C isotope labeled at the position of 15 carbon atom
¹⁸ O-retinal	retinal dehyde with ¹⁸ O isotope labeled on the oxygen atom of carbonyl group
FT-IR	Fourier Transform Infrared Spectroscopy
RR	Resonance Raman Spectroscopy
CCD	Charge Coupled Device

Chapter 1

INTRODUCTION

I. General Intruduction

The term "Vitamin A" is used for retinoids that exhibit the biological activity of retinol. Figure 1.1 shows the structural formulas of β -carotene, the precursor of retinoids and some naturally occurring retinoids. It is well known that Vitamin A and its metabolites play an important role in diverse biological systems, such as in human vision, fetal development and the regulation of proliferation and differentiation of cells throughout life. These fat-soluble organic compounds, however, can not be synthesized endogenously by humans but must be obtained from dietary sources. Vitamin A and its derivatives are also quite poorly soluble in water (Szuts, and Harosi, 1991). Therefore, a well-regulated transport system has been suggested to deliver the retinoids from the intestine to the various target tissue systems where they are stored or utilized. It has been generally believed that a number of different water soluble proteins that are devided into two classes bind these hydrophobic molecules and mediate their delivery (Blomhoff, *et al.*, 1990). These proteins bind retinoids in distinctly different ways as

determined by their physiological functions. However, the binding mechanisms for different retinoids are not yet already understood, i.e. through a linkage of Schiff base ($C=N$), protonated Schiff base ($C=NH^+$), or merely in a form of the hydrogen-bonded aldehydes or alcohols. Moreover, other bonding interactions are likely. It is our fundamental goal to determine the mechanism of the interaction between retinoids and their corresponding proteins, including the conformational changes of retinol ligands due to binding, as well as binding preference of one retinol isomer over others.

We have chosen three members of these two classes of the retinoid binding proteins: serum retinol binding protein(RBP), cellular retinol binding protein, type I (CRBP-I), and cellular retinaldehyde binding protein (CRALBP). These proteins have different physiological functions and differ substantially in their binding with vitamin A and its derivatives (see Table 1.1) (Saari, *et al.*, 1984). Some X-ray structures of these binding proteins have been resolved, which are very helpful in our data analysis. Therefore they offer an excellent set for vibrational spectroscopic studies. RBP is a single polypeptide chain of 182 amino acids with a molecular weight of about 21 kD and essentially binds equally well the various isomers of retinol (*trans*, 11-*cis*, etc.) as well as other retinoids. Examples of this are retinal and retinoic acid (Blaner, 1989). In blood, RBP circulates as a 1:1 molar complex with another serum protein, transthyretin (TTR), and helps transport retinol from its stores in the liver to target organs. A published 2 Å X-ray structure shows an anti-parallel β -barrel surrounding the retinol ligand (Cowan, *et al.*, 1990). The λ_{max} 's of the bound all-*trans* retinol

and retinal have no shift from those at approximate 325 and 380 nm in CCl₄ solution.

CRBP-I, a protein of 14 kD with 134 amino acids, is found in the small intestine of adult rat. This protein shows a strong binding preference for retinol of the retinoids over retinal as well as a preference for *trans* over *cis* isomers. Its function has not been established as yet. It has been proposed that CRBP-I functions in the delivery of retinol intracellularly to the enzyme lecithin:retinol acyltransferase(LRAT) for esterification and storage (Ong, *et al.*, 1987, 1988; Saari, and Bedberg, 1988; Yost, *et al.*, 1988). The λ_{max} 's of the bound retinol and retinal in CRBP-I are 348 nm and 408 nm, about 25 nm shifted towards the red compared to those in CCl₄ solution. A 2.1 Å retinol/CRBP-I X-ray structure has been published (Cowan, *et al.*, 1993). It shows that all-*trans* retinol has a planar conformation with its alcohol group hydrogen bonding to the side chain of glutamine 108. This local interactions of glutamine 108 explain CRBP-I's preference for binding retinol rather than retinal, which is ascertained in our vibrational spectroscopic studies.

The third protein, CRALBP, is of the most interest. This single amino-terminally acetylated polypeptide chain contains 316 residues, corresponding to a molecular weight of 36 Kd (Crabb, *et al.*, 1988). This protein binds specifically the 11-*cis* and 9-*cis* isomers of retinal, however no complexes were detected with 13-*cis* and all-*trans*-retinaldehyde (Saari, and Bredberg, 1987). Notably, CRALBP has only been found in tissues that respond to light (retina and pineal) and can discriminatingly select 11-*cis*-retinaldehyde from a mixture of retinoids precluding it from photoisomerization. This

suggests a possible role in the generation of 11-*cis*-retinoids in the visual cycle. The λ_{max} 's of the CRALBP complex with 11-*cis* and 9-*cis* retinal are at 425 nm and 405 nm, respectively, far to the red of about 380 nm absorption of these molecules in CCl₄ solution. These differences in binding specificity and in absorption maximum amongst these protein-retinoid complexes are the result of varying interactions between the chromophore and the protein.

Raman scattering is a process of inelastic collision between photons and matter. Raman spectroscopy is concerned primarily with the vibrational energy level transitions of the molecule. Thus it provides a sensitive probe to detect detailed information on the vibrational motions of atoms in molecules. Equipped with sophisticated Raman spectroscopic facilities in our laboratory, we were able to investigate the binding mechanism between the retinol binding proteins and their corresponding specific ligands through the analysis of changes in vibrational modes of these prosthetic groups. Resonance Raman spectroscopy provides a very good tool to obtain the vibrational spectra of these chromophores inside protein pocket without interference from the vibration of the amino acid environment. In principle, resonance Raman scattering results from the energy of laser beam coinciding with that of an electronic transition in a molecule. If the energy of laser light is close to, but not on an electronic excitation level of the molecule, the process is called pre-resonance Raman scattering. Compared to the intensity of classical Raman scattering, the intensity due to Raman resonance is considerably enhanced by a factor of about 2000, depending on various excitation lights. For the holo-retinol binding proteins, if

appropriate laser lines are chosen to be in resonance with the absorption of chromophores, but still removed from the absorption of proteins, we can obtain very strong Raman scattering signals basically from ligands with minimal protein background.

There exists, however, a major difficulty in directly applying Resonance Raman spectroscopy due to sample isomerization in our studies. The molecules of Vitamin A and its derivatives are photolabile, or sensitive to light, and consequently isomerize to different conformations. For instance, the 11-*cis* retinal will isomerize to the all-*trans* or other retinal conformers upon absorbing a photon. Usually, two techniques are employed to attack the difficulty of light induced isomerization. One is molecular flow resonance Raman technique (Callender, *et al.*, 1976). In this method, a molecular flow is imposed to run transversely through the Raman irradiating laser beam along a capillary tube with sufficient velocity to ensure that any given molecule moves out of the probe section before there is any significant probability of photon absorption. Thus the Raman signal detected is essentially only the nonphotoisomerized pure starting material. However, this method needs relatively large amount of samples. Since our samples are very limited, it is not feasible for us to apply this molecular flow technique. The second approach is the above mentioned pre-resonance Raman spectroscopy. This technique utilizes far-red laser line to excite Raman scattering on a stationary sample, which is removed enough from the absorption center of chromophores, insuring no significant isomerization but retains a resonance effect. Even though the resonance effect is attenuated, the comparison of

the chromophore Raman signal over that of the protein is still considerable. We tested this method on 11-*cis*-retinal ($\lambda_{\text{max}}=380$ nm) with 676 nm line and its PSB ($\lambda_{\text{max}}=440$ nm) with 752 nm, and found no difference between our results and those obtained by flow technique mentioned above. This approach has also been applied by other groups and no evidence of isomerization was observed (Curry, *et al*, 1982; Smith, *et. al.*, 1985). Furthermore, the amount of samples needed for this method is only about 20 to 30 μL . For this reason, we employed the pre-resonance Raman technique to acquire Raman scattering data.

A number of experiments have been performed, including measurements of Raman spectra of holo-proteins, apo-proteins if possible and retinoid model compounds in solution. With the aid of isotopic labelings and difference Raman spectroscopy, we were able to extract detailed information of retinoid conformational changes that accompany the binding of these proteins from solution.

II. Retinoid Compounds

Retinal compounds are the subjects we mainly focus on in the investigation of the binding mechanisms of protein/retinal ligand interactions. Detailed information about chromophore structure and chromophore-protein interactions is in principle obtainable from the resonance Raman or preresonance Raman and infrared data. The well established analysis of the biologically significant retinal isomers has brought this promise nearer to fulfilment by providing a framework for the analysis of ligand spectra. For example, comparison of observed frequencies, intensity, and isotopic shifts of

the aldehydes with those of visual pigments has pointed to systematic discrepancies which can be attributed to protein perturbations (Callender, *et al.*, 1987, 1984, 1978, 1977, 1974; Eyring, *et al.*, 1982). In our work, we utilized frame work of these retinal isomers and their derived model compounds to analyze the experimental results. Therefore, we take a few moments to understand these retinoids.

Conformations of various isomers of retinoids, their Schiff bases ($X=N$), and protonate Schiff bases ($X=NH^+$).

Retinal compounds are characterized by the geometry of their polyene chains and the terminal groups which play essential roles for their binding to proteins, such as through formation of hydrogen bonds, Schiff base or protonated Schiff base (See Figure 1.2). Basic conformations of retinoids are defined by the bending position relative to double bonds along their polyene tails as shown in Figure 1.1. For example, 11-*cis* retinal forms *cis* about the $C_{11}=C_{12}$ double bond like $/C_{11}=C_{12}\backslash$, whereas all-*trans* retinal has a *trans* geometry as $/C_{11}=C_{12}/$. These configurationally various compounds can convert to one another upon absorbing a photon, namely isomerization. This photolability usually induces protein conformational changes, which consequently activates another biological process, e.g. the retinal regulation of large global conformational changes of the apo-protein in the induced purple membrane triggered by very weak photoenergy (Gibson, and Cassim, 1987). However, this may also cause a very serious problem in the resonance Raman spectroscopic study as mentioned earlier.

Vibrational Analysis of Retinal Isomers, Schiff Base and Protonated Schiff Base.

Isotopic substitution combined with normal mode calculations is the classical method of vibrational analysis. Furthermore, the Raman spectrum is significantly simplified because pre-resonance excitation permits the selective enhancement of chain rather than ring vibrations. In our studies of the characterization of interactions between protein and bound retinal ligand, the interpretation of Raman spectra are performed in the light of well established framework on the analysis of retinal isomers and their derived compounds, such as Schiff base and Protonated Schiff base that may be formed as linkages between retinal ligands and their corresponding binding proteins (Callender, 1982; Curry, *et. al.*, 1985).

Retinoids have their inherent absorption spectra, which are subject to different binding protein environments. But their Raman spectra can provide in a great detail of information about their vibrational alterations upon binding to the proteins. Retinoids consist of a β -ionone ring, a methylated polyene chain made up of repeating isoprenoid units, and a terminal group which may be modified when bound to the binding proteins. In resonance or pre-resonance Raman spectra, the vibrations of the β -ionone ring are weak or absent, because the ring participates only slightly in the resonant $\pi \rightarrow \pi^*$ electronic transition. Those β -ionyl vibrations are generally insensitive to chain configuration or end-group modifications. Usually, retinal Raman spectra are analyzed in terms of three spectral regions: *ethylenic-Schiff base* region, *finger-print*

region and *HOOP* (hydrogen-of-out-plane) modes. Figure 1.3 demonstrates the Raman spectra of all-*trans* retinal, its SB and PSB. As it can be seen clearly in the picture, the ethylenic-Schiff base regions of the three spectra have well defined patterns for all-*trans* isomer and its related compounds. We now discuss these three regions separately in the following.

Ethylenic - Schiff base region (1500-1700 cm^{-1})

The stretching modes of C=C, C=O, C=N and C=NH⁺ double bonds are found from 1500-1700 cm^{-1} (see Figure 1.4). The C=O stretch bands of all of the retinal isomers are located at about 1664 cm^{-1} , practically constant in wavenumber and intensity in the spectra. This mode is only slightly coupled with the C₁₅H and C₁₄H rocks. However, the C=O bands will shift about 10 to 40 cm^{-1} when bound to relevant binding proteins through hydrogen bonding, usually with a concomitant broadness in band width due to a wide energy distribution. The most prominent feature of the Raman spectra of retinoids is the intense band between 1570 and 1580 cm^{-1} , due to the predominantly in-phase stretching vibration of the conjugated double bonds through the π -electron system. The position of this band is linearly correlated to the λ_{max} of retinal's absorption band, so that a shift to lower frequency represents a shift to a higher λ_{max} (Kakitani, *et al.*, 1983). The C=N stretching mode has been found between 1620-1650 cm^{-1} , depending the state of protonation of the Schiff base (Oseroff, and Callender, 1974). Upon protonation, Schiff base band shifts up to higher frequency due to the coupling with the C=N-H bend, which makes an important contribution to the C=N

stretch and pushes it to the higher frequencies (see Figure 1.3, all-*trans* retinal Schiff base (B), and protonated Schiff base (C)). In vivo, for instance, the Schiff base linkage between all-*trans* retinal and bacteriorhodopsin M412 is at 1621 cm^{-1} , whereas the protonated Schiff base bond between 11-*cis* and rhodopsin is at 1658 cm^{-1} .

Finger-print region ($1000\text{-}1450\text{ cm}^{-1}$)

The so-called fingerprint bands, between $1000\text{-}1450\text{ cm}^{-1}$, result from C-C single bond stretches and C-H in plane bending motions arising both from intra-polyene chain carbons and C-CH₃ vibrations (see Figure 1.5). This region has been called the "finger-print" region because of its marked sensitivity to polyene isomer configuration. The conjugated single bond stretching coordinates are responsible for most of the Raman intensity in the retinal finger-print region. Since these stretches are coupled with each other through the π -electron system, they mix considerably and exchange intensity when they are close together in frequency. This coupling extends along the entire conjugated chain, so that spatially distant stretches can still interact significantly. The stretching normal modes are nonetheless quite localized, because the chain methyl groups profoundly alter the environment of the adjacent C-C bonds. The C-C-H rocking vibrations of the seven chain hydrogen atoms of retinal molecules appear in the upper part of the finger-print region, strongly coupled with chain C-C stretches.

Hydrogen-out-of-plane (HOOP) mode region ($750\text{-}1000\text{ cm}^{-1}$)

The out-of-plane wags (see Figure 1.6) of chain hydrogen atoms couple strongly with the adjacent C-C torsions and with the wags of adjacent carbon substituents. Both wag-torsion and wag-wag couplings are strongly dependent on the π -bond order of the C-C bond. Each of the hydrogen wags of retinal molecules interacts with both an adjacent C-C and a C=C torsion, so that the net wag-torsion coupling is nearly the same for all the vinyl wags. Hydrogen wags should not be enhanced in the preresonance Raman spectrum of a strictly planar chromophore. Raman intensity in these bands indicates a distortion from the planar geometry in either the ground or the resonant excited state. A quantitative calculation of this strain angle along the chromophore's polyene chain has been developed by Warshel *et al.* (cf. Warshel, and Dauber, 1977) as the following where the dimensionless displacement Δ is related to a displacement along the internal wag coordinate δ or torsional angle (radians) by

$$\Delta_{\text{HOOP}} = \left(\frac{4\pi^2 c \omega \mu}{h} \right)^{1/2} \delta \quad (1.1)$$

where, ω is the mode frequency in wavenumbers and μ is the electronic transition moment. Since resonance Raman intensities are also proportional to other factors, including the electronic transition intensity, we must ratio the HOOP intensity to some reference mode whose Δ is known to get a meaningful value for Δ_{HOOP} .

In calculations, I_{HOOP} is usually normalized to the C=C ethylenic mode at about 1580 cm^{-1} . Thus, we have

$$I_{\text{HOOP}} \sim (\Delta \text{HOOP})^2 \quad (1.2)$$

and

$$I_{\text{HOOP}}/I_{\text{C=C}} = (\Delta \text{HOOP}/\Delta \text{C=C})^2 \quad (1.3)$$

III. Retinoid Binding Proteins-RBP, CRBP-I, and CRALBP

The physiological functions of retinoid binding proteins in the transportation of retinoids are various. Retinol binding protein (RBP) is the only retinoid-specific binding protein that has been found in plasma. It is synthesized in the hepatocytes, where it accumulates until the binding of its ligand triggers its secretion (Goodman, "The Retinoids," 1984). RBP can bind a number of retinoids. Circulating in the plasma, RBP is found complexed to transthyretin. The formation of this protein complex prevents the lower molecular weight retinol binding protein from being filtered through the kidney glomeruli (Kanai, *et al.*, 1968). Upon delivery of the retinol, however, the RBP-transthyretin complex dissociates and the RBP is filtered and degraded. Thus, each RBP molecule transports just a single retinol molecule before it is degraded. An X-ray crystallographic structure of RBP has been published by S. W. Cowan, *et al.* (Cowan, *et al.*, 1990). It shows that, inside the hydrophobic binding pocket, the retinol molecule is completely encapsulated by the β -barrel core, with β -ionone ring in the center and the isoprene chromophore stretching along the barrel axis almost to the solvent surface (see Figure 1.7).

CRBP-I is the predominant intracellular retinoid-binding protein (CRBP) in most tissues. It is also most concentrated in the liver, lung, kidneys, epididymis and testis. Unlike RBP, CRBP-I does not bind to transthyretin. This protein binds all-*trans* retinol and

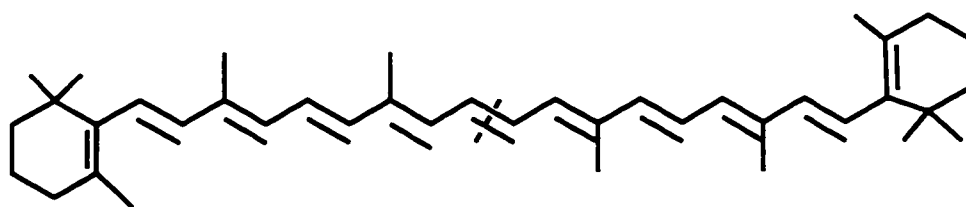
all-*trans* retinal, but is more specific for retinol (Li, *et al.*, 1991). CRBP-I is able to transfer the vitamin A specifically into the cellular nucleus to specific binding sites on the chromatin, without itself remaining bound to the chromosomal material (Takase, *et al.*, 1979). As present, the biological functions of CRBP-I have not been clearly defined. There is evidence that CRBP-I can mediate the uptake and esterification of all-*trans* retinol by bovine retinal pigment epithelium (Ottonello, *et al.*, 1987). Other studies have shown that CRBP-I can facilitate the transfer of retinol to the nucleus (Takase, *et al.*, 1979; Liau, *et al.*, 1981, 1985). An X-ray study indicates that CRBP-I also has a β -barrel structure but not that the way CRBP-I binds all-*trans* retinol differs from that for RBP, with the hydroxyl group is innermost, at the center of the barrel (Cowan, 1993, see Figure 1.8).

Cellular retinaldehyde binding protein (CRALBP) is a vision-related intracellular retinoid-binding protein. The physiological role of CRALBP is not yet fully understood. However, several lines of evidence suggest that the protein is a functional component of the visual cycle. First, CRALBP has only been found in retina and pineal, both light-sensitive tissues, but not in any other tissues examined so far (Crabb, *et al.*, 1988). Second, the endogenous ligands bound by CRALBP are 11-*cis* retinol and 11-*cis* retinaldehyde, retinoids that are only known to function in the visual process. Third, CRALBP interacts specifically with the visual cycle enzymes 11-*cis* retinol dehydrogenase of retinal pigment epithelium, catalyzing the reduction of 11-*cis* retinaldehyde bound to CRALBP, and retinyl ester synthase, yielding retinyl esters (Saari, *et al.*, 1982). Finally, CRALBP

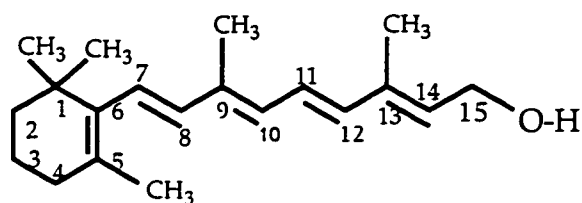
is able to select 11-*cis* retinaldehyde, the isomer form found in rhodopsin, from a mixture of vitamin A stereoisomers, where it protects it from photoisomerization, suggesting a role for the protein in the generation of 11-*cis* retinal. No X-ray diffraction structure study has been done. Therefore, our Raman spectroscopic study is crucial to characterize the mechanism of interaction between CRALBP and its retinoid ligand.

Table 1.1 Retinoid-binding proteins selected for study and their biological features.

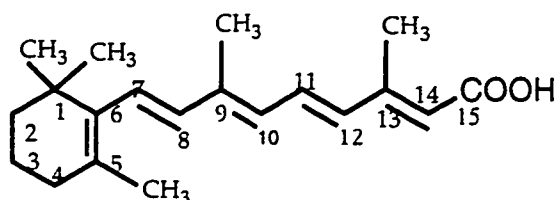
Protein	RBP	CRBP-I	CRALBP
Amino residues	183	134	316
MW (kD)	21	16	36
Specificity	various	retinol, retinal	11- <i>cis</i> retinal, 9- <i>cis</i> retinal
Function	Blood plasma transport	Donor for LRAT reaction, intracellular transport	Enzymatic reactions in the visual cycle



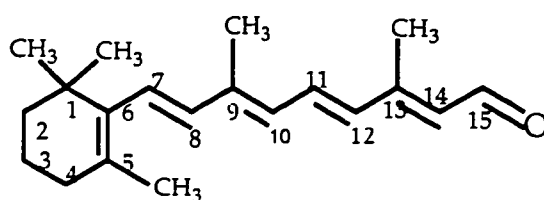
β -carotene



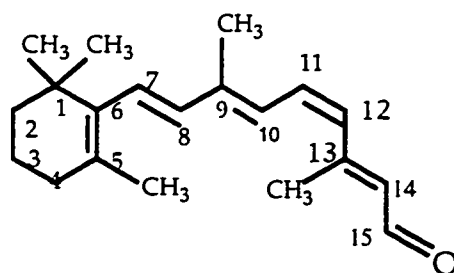
all-trans retinol



all-trans retinoic acid



all-trans retinal



11-cis retinal

Figure 1.1 β -Carotene and some naturally occurring retinoids.

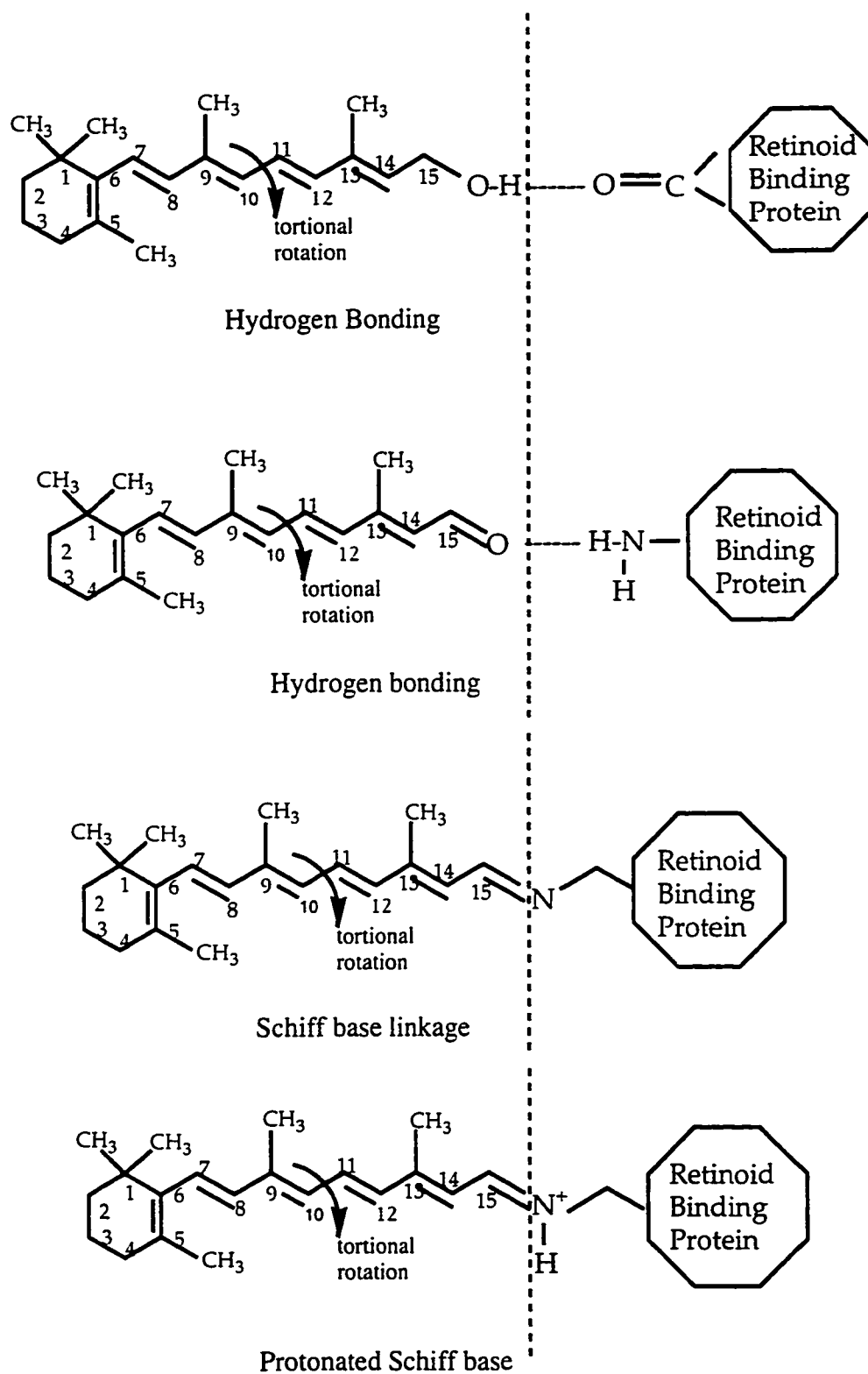


Figure 1.2 Binding schemes between retinoids and their corresponding binding proteins.

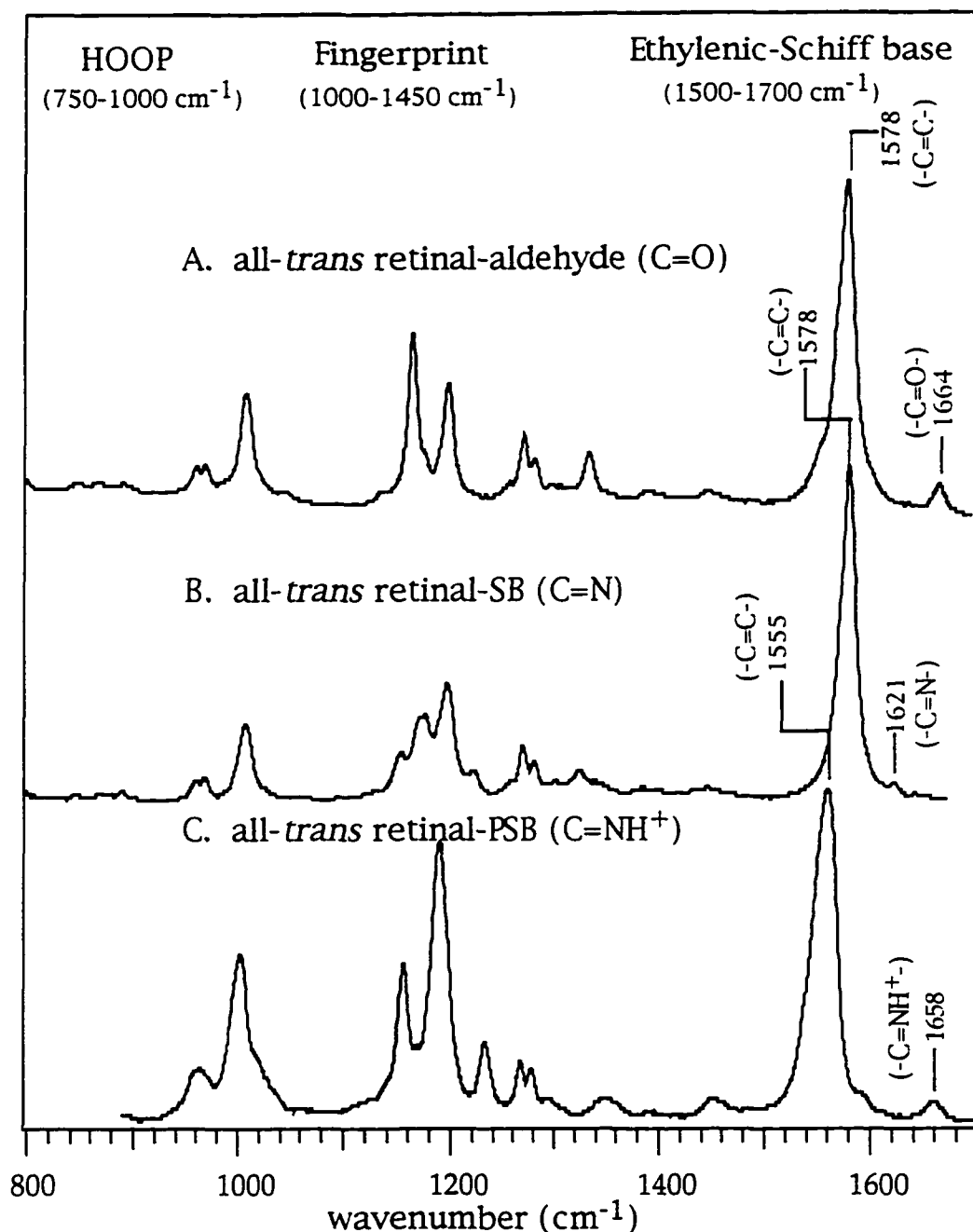
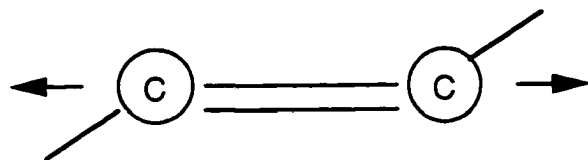
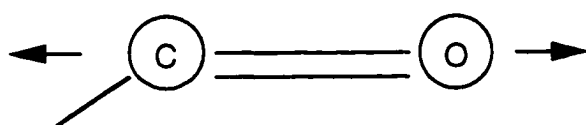


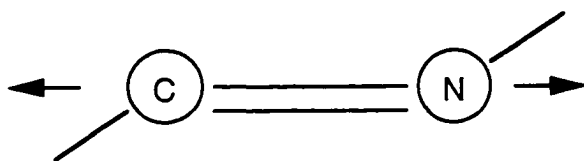
Figure 1.3 Raman spectra of all-*trans* retinal and its terminal group modified derivatives, Schiff base (SB) and Protonated Schiff base (PSB). As shown in the picture, both of the ethylenic and fingerprint regions are uniquely defined, which are used to characterize the binding mechanism of protein/ligand complex.

Ethylenic region(1500 - 1700 cm⁻¹) modes

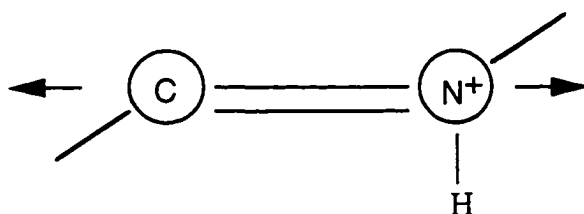
C = C stretching mode



C = O (carbonyl) stretching mode

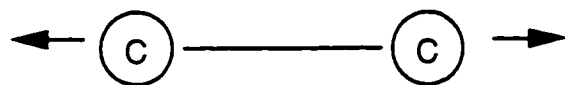


C = N (Schiff base) stretching mode



C = NH⁺ (Protonated Schiff base) stretching mode

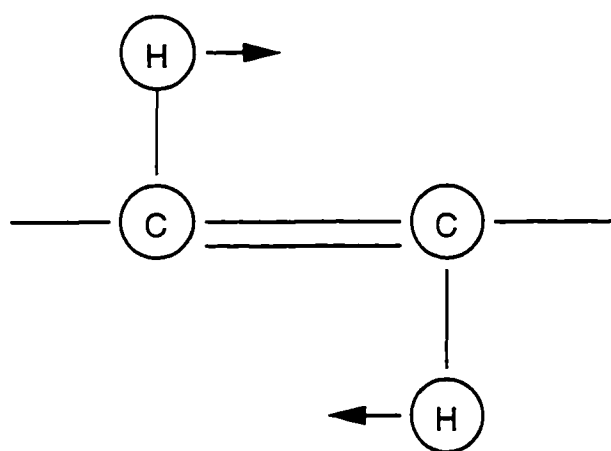
Figure 1.4 Vibrational modes that fall in the
Ethylenic - Schiff base region.

Finger - print region (1000 - 1450 cm^{-1}) modes

C -- C stretching mode



C --Methyl group stretching



Hydrogen in plane bending

Figure 1.5 Typical group modes of vibrational motion that define the fingerprint region for different retinoid compounds.

**Hydrogen-out-of-plane wagging mode
(800 - 1000 cm^{-1}) mode**

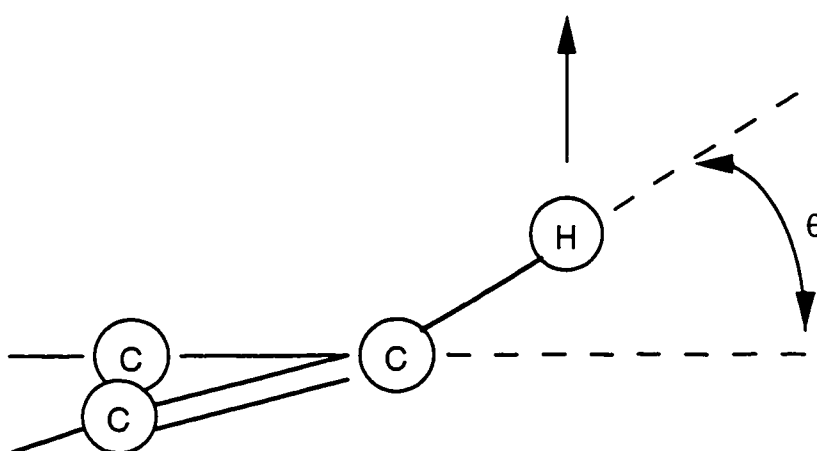


Figure 1.6 The hydrogen out of plane motion. Angle θ is defined by C-H bond and the plane formed by -C-C=C-. Change of θ indicates departures of linearity of the polyene chain of retinoids.



Figure 1.7 X-ray structure of the complex of RBP/*all-trans* retinol at resolution of 2 Å. The retinol ligand is surrounded by an anti-parallel β -barrel, with its terminal group sticking out of the binding pocket.



Figure 1.8 2.1 A X-ray structure of CRBP-I/all-*trans* retinol complex. The binding pocket is composed of 9 β -strands. Unlike in RBP, all-*trans* retinol lies in the binding site with its terminal group innermost, hydrogen bonded to Gln108 residue.

Chapter 2

BASIC THEORY AND INSTRUMENTATION OF RAMAN SPECTROSCOPY

I. The Raman Effect

When electromagnetic radiation of energy content $h\nu$ irradiates a molecule, the energy may be transmitted, absorbed, or scattered. In the Tyndall effect the radiation is scattered by particles (smoke or fog, for example). In Rayleigh scattering, molecules scatter the light. No change in wavelength of individual photons occurs in either Tyndall or Rayleigh scattering. However, it is possible that during a process of the interaction between a photon and molecule the energy of incoming photons can be altered as shown in Figure 2.1. The upward-pointing arrows indicate the combination of a photon with the molecule, whereas the downward-pointing arrows indicate the release of a photon after a very short time interval, less than 10^{-13} second for Raman process (virtual process). The lengths of the upward-pointing arrows are proportional to the frequencies of the incident light and those of the downward-pointing arrows are proportional to the frequencies of the scattered light. Upon

interacting with the incident photon, the molecule is brought up to a virtual energy state, the molecule emits light and returns to its ground electronic state. If the length of the upward-pointing arrow is equal to that of the downward-pointing arrow, Rayleigh scattering takes place. On the other hand, if the length of the downward-pointing arrow is shorter (Stokes process) or longer (anti-Stokes process) than that of upward-pointing arrow, the frequencies of the scattered light are thus shifted from that of the incident light. This phenomenon is called the Raman effect. In principle, the positions of Raman peaks are properties solely of the electronic ground state, while the Raman intensities are functions of both ground and excited state electronic structures.

The Raman effect is a very useful tool in the biochemical studies as it can provide a probe to monitor the vibrational structures of biomolecules.

II. Raman and Resonance Raman Spectroscopies

The Raman spectrum is a vibrational spectrum of molecule. Raman spectra give detailed information on the vibrational motions of atoms in molecules, and, because these vibrations are sensitive to chemical changes, the vibrational spectrum can be used as a monitor of molecular chemistry. Essentially, the same kind of vibrational motion that produces the peaks in Raman spectra also produces the "peaks" (actually, absorption maxima) in infrared spectra. However, the physics of the Raman effect and the infrared absorption process are quite different. Already said above, the physical origin of Raman scattering lies in inelastic collisions between molecules and photons.

In inelastic collision the photon exchanges energy with the molecule, resulting in the change of the wavelength of the scattering light. The shift in frequency of the incident light, hence the change in energy, is equal to the energy difference between the vibrational states of the molecule. When the excitation light is selected close to a particular absorption band of a biomolecule, the cross-sections of Raman modes coupled to this absorption band are greatly increased. As a result, the Raman scattering will be significantly enhanced, which is defined as resonance Raman effect. Compared to the intensity of classical Raman scattering, the intensity due to Raman resonance can be increased by many orders of magnitude. Resonance Raman spectroscopy provides a very good tool to obtain the vibrational spectra of the chromophores inside protein pockets without interference from the vibration of the amino acid environment. Since the vibrations of the protein are not seen, complicated preparation procedures are not needed and, in fact, in situ experiments are possible.

Raman spectroscopy has its own striking merits in biochemical investigations over X-ray crystallography and Infrared (IR) spectroscopy (Carey, 1982). For instance, Raman spectra may be obtained for molecules in aqueous solutions which is similar to the real physiological conditions, in contrast to the X-ray studies where crystallized biomolecules are measured. Furthermore, the interactions that take place between molecular groups, such as hydrogen bonding, affect the force constants of certain bonds and, hence, vibrational frequencies. Thus one can probe the energies of hydrogen bonds directly by examining shifts in the frequencies of

certain bands from so-called Badger-Bauer relationships (Badger, and Bauer, 1937). This method is more accurate than probes of protein structure such as X-ray crystallographic studies. A 0.01 Å change in bond length will be easily detected by 5 cm⁻¹ shift in Raman spectroscopic study. However, sometimes fluorescence gives rise to a big problem in practicing Raman spectroscopy, and special treatments of samples have to be performed. Furthermore, the high photon flux of the laser beam can produce unwanted photon chemical effects, such as photoisomerization of retinoid compounds.

III. Normal Modes of Vibration and Normal Coordinates

Since atomic nuclei are massive compared to electrons, it is a good approximation to describe the dynamics of a molecule in terms of the motions of the nuclei alone. Each nucleus requires three coordinates to describe its position (e.g., in a Cartesian coordinate systems, x, y, and z). Thus each nucleus has three independent degrees of freedom, and for a molecule containing N atomic nuclei the total number of degrees of freedom is 3N. However, we are interested in only the vibrational dynamics of the molecule and we can subtract out the overall translational and rotational motions since these do not affect the internal vibrations. The center of gravity of the molecule requires three coordinates to define its position, and thus there are three independent degrees of freedom of motion associated solely with the translations of the center of gravity. Similarly, when a nonlinear molecule is in its equilibrium configuration, it requires three additional coordinates to describe its rotational motion about the center of gravity. A nonlinear molecule, therefore, has three

independent rotational degrees of freedom. A linear molecule, however, has only two rotational degrees of freedom. Taking these facts into account that translations of the center of gravity and rotations about this point occur without changing the shape of the molecule, and the internal degrees of freedom, which are associated with vibrational motions, change the shape of the molecule without moving the center of gravity or rotating the molecule, we subtract the translational and rotational degrees of freedom from the original total $3N$ and see that a nonlinear molecule has $3N - 6$ internal degrees of freedom and a linear molecule has $3N - 5$ internal degrees of freedom.

As one can imagine, the vibrational motion of a complex molecule composed of N atoms is almost impossible to be described as any order or pattern. Fortunately, however, this complicated vibrational motion of an N atom molecule can be broken down into simple components known as the normal vibrations, or normal modes. For example, the $3N - 6$ internal degrees of freedom of a nonlinear molecule correspond to $3N - 6$ independent normal modes of vibration. In each normal mode all the atoms in the molecule vibrate with the same frequency, and all the atoms pass through their equilibrium positions simultaneously. If we make an approximation that the potential energy of the vibrational motions is proportional to the square of the displacement from the nuclear equilibrium position, in each normal mode under this assumption, every mass performs simple harmonic motion with the same characteristic frequency ν_{vib} . Figure 2.2 is an example of normal modes with CO_2 .

Since it is linear, CO_2 , on the basis of the above, has $3 \times 3 - 5 = 4$ degrees of freedom and hence 4 normal modes.

The coordinate systems of normal modes of vibration can be described using either Cartesian coordinates or internal coordinates involving change in bond lengths or bond angles. However, another coordinate system, namely the normal coordinate, is often chosen due to its particular advantages. In this system, each normal mode of vibration is characterized by a single coordinate called the normal coordinate Q . Q varies periodically with time, and as one normal coordinate vibrates, every Cartesian displacement coordinate and every internal coordinate vibrates, each with an amplitude in a specified proportion to the amplitude of the normal coordinate. The proportionality factors relating the amplitudes are such that the resulting motion is a normal mode. The mathematical advantage arises from the fact that each normal mode may be excited without affecting any of the other normal modes. Each normal coordinate makes a separate and independent contribution to the kinetic and potential energy of the system. This allows the contribution from each normal mode to be treated separately.

Even though, however, theoretically all the normal modes of vibrations of a molecule can be calculated, for large biochemical molecules, such as proteins, this work is extremely formidable and the results are hardly applicable to spectroscopic studies. As a matter of fact, it is sometimes found that certain normal modes for a polyatomic molecule are dominated by the motions of just a few neighboring atoms. In other words, although in each normal mode of a polyatomic molecule every atom undergoes a periodic oscillation

with the same frequency ν , the amplitudes of each oscillation are generally different. The vibrational modes of these chemical functional groups are called group frequencies. Group frequencies find their maximum utility when they are relatively easy to be identified from other Raman bands and when they vary with environmental interactions. The concept of group frequencies is the basis of Raman spectroscopic analysis in our studies.

An example of how group frequencies may be useful is when they are applied in the pre-resonance Raman spectroscopic study on the interactions between retinol ligands and their corresponding binding proteins. The carbonyl C=O stretching mode of retinal ligands shows intense features in their Raman spectra near 1660 cm^{-1} . This band is subject to hydrogen bonding environment. By monitoring the feature of this band, we will be able to tell the type of protein-chromophore interaction, as well as the local binding strength.

IV. (Resonance) Raman Intensities

In order to gain further insight into resonance enhancement, we apply a quantum mechanical approach in which excited electronic states become explicitly involved. A usual application of this would be to obtain the vibrational spectrum of a colored prosthetic group, for instance, a retinal chromophore, in a protein without interference from the vibrations of the amino acid environment.

For randomly oriented molecules, the total intensity of the scattered light resulting from a molecular transition between states m and n is

$$I = K(\nu_0 \pm \nu_{mn})^4 I_0 \sum_{\sigma\rho} |(\alpha_{\sigma\rho})_{mn}|^2 \quad (2.1)$$

where, $\alpha_{\sigma\rho}$ is the transition polarizability tensor,

$$(\alpha_{\sigma\rho})_{nm} = \frac{1}{h} \sum_r \frac{\langle n|\mu_\rho|r\rangle\langle r|\mu_\sigma|m\rangle}{\nu_m - \nu_0 + i\Gamma_r} + \frac{\langle n|\mu_\sigma|r\rangle\langle r|\mu_\rho|m\rangle}{\nu_m + \nu_0 + i\Gamma_r} \quad (2.2)$$

and K is a constant.

In these expressions the molecule is considered to be in the initial molecular state m . A light of frequency ν_0 and intensity I_0 causes the transition to a state n and scattering light of frequency $\nu_0 \pm \nu_{mn}$. The sum over index r covers all of the quantum mechanical eigenstates of the molecule, h is Planck's constant, and Γ_r is a damping constant which takes into account the finite lifetime of each molecular state. The $\langle n|\mu_\rho|r\rangle$, etc., are the amplitudes of the electric dipole transition moments, where μ_ρ is the electric dipole moment operator along direction ρ .

More specifically, we may break the molecular states $|m\rangle$ and $|n\rangle$ down into their electronic and vibrational components as the following approximation,

$$|m\rangle = |g\rangle|i\rangle, \quad |n\rangle = |g\rangle|j\rangle, \quad |r\rangle = |e\rangle|v\rangle \quad (2.3)$$

where g is the ground electronic and e the excited electronic state, i, j are vibrational states associated with g , and v is a vibrational state associated with e . By substituting Eq. 2.3 into 2.2, the component of the polarizability tensor, is expressed as

$$(\alpha_{\sigma\rho})_{mn} = A + B \quad (2.4)$$

where

$$A = \frac{M_e^p M_e^o}{h} \sum_v \frac{\langle j|v\rangle\langle v|i\rangle}{\nu_{vi} - \nu_o + i\Gamma_v} \quad (2.5)$$

and

$$B = \frac{M_e^p (\partial M_e^o / \partial Q)^o}{h} \sum_v \frac{\langle j|v\rangle\langle v|Q|i\rangle}{\nu_{vi} - \nu_o + i\Gamma_v} \quad (2.6)$$

M is the electronic transition moment as a function of normal coordinate and $\langle i|v\rangle$ etc. are overlap integrals between vibrational wave functions of the ground states and excited states, g and e .

The first term of the above expression, the Albrecht A-term, is the predominant term in the Raman tensor in the resonance case for the retinal chromophore (Warshel, and Dauber, 1977; Doukas, *et al.*, 1978a).

It can be shown that in a near resonance case, if the damping factor Γ can be neglected, relative Raman intensity I of two modes i and j can be expressed as,

$$\frac{I_i}{I_j} \approx \left(\frac{\Delta_i \nu_i}{\Delta_j \nu_j} \right)^2 \quad (2.7)$$

where $\Delta_{i,j}$ are proportional to the equilibrium shift upon excitation of the normal mode i, j and $\nu_{i,j}$ are their vibrational frequencies (Kakitani, *et al.*, 1979). This relationship is used to calculate the torsional angle along the retinal polyene tail by the measurement of HOOP mode intensity.

It is important to emphasize that the frequency positions in Raman and resonance Raman spectra are a property solely of the

electronic ground state. While Raman peak intensities may be correlated with ground state properties, intensities under resonance conditions can only be understood by considering the excited electronic state of the molecule. (Carey, 1982).

V. Isotopic Labelings

In the analysis of vibrational spectra, isotopic substitution is often applied at certain positions in order to relate the Raman peaks to the vibrational modes of biochemical molecules. The success of the application of isotopic labelings is based on the assumption, which is generally valid, that the force constants are unaltered by isotopic substitution, therefore the shift in observed frequency can be attributed principally to mass effects which is shown in the following relationship, a very simple relationship for a diatomic oscillation,

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \quad (2.8)$$

where k is the force constant, and μ is the reduced mass, which, upon isotopic replacement, will be changed, and so will the vibrational frequency ν . The resulting frequency shifts of the normal modes provide a good measure of the degree to which the substituted local coordinates' contribute to each normal mode of the original molecule (Curry, *et al.*, 1985). In our work, ^{13}C and ^{18}O isotopic labelings to the retinoid compounds are employed as will be discussed in later chapters. For instance, the unlabeled all-trans retinal carbonyl $\text{C}=\text{O}$ stretching mode is at 1664 cm^{-1} in CCl_4 solution. It will be observed down shifted to 1629 cm^{-1} (see Figure 2.3) upon isotopic editing with ^{13}C , as expected according to the Eq. 2.8.

VI. Methodology and Instrumentation

i). Difference Raman Spectroscopy

In pre-resonance Raman spectroscopy, the Raman spectra of chromophores bound to protein are generally acquired on the background of protein environment. Unfortunately, however, sometimes the contributions from the protein are so strong that they may interfere with the identification of chromophore peaks and sometimes these bands attributable to the protein are impossible to be removed by subtracting the spectrum of the apo-protein. For instance, CRALBP is unstable in its apo form and therefore its Raman spectrum is unobtainable. Unfortunately the protein background is often strong enough to obscure the bands of interest in CRALBP. The issue can be solved by Raman difference spectroscopy and isotope editing (cf. Callender, and Deng, 1994; Yue, *et al.*, 1989). The Raman spectra of protein complexed with the retinal ligand and that with isotopically labeled retinal ligand are acquired under the same conditions. The isotopic substitution on the ligand has no effect on the protein. Thus, performing numerical subtraction between the two spectra, we are able to obtain a derivative spectrum which only contains the peaks related to the labeled atom and its unlabeled counterpart of the bound ligand. The protein background is completely canceled out, as well as those of the ligand which are unaffected by the isotopic substitution.

As the difference spectrum is small, care should be taken in order to reduce the influences of different optical properties for the different samples and the environmental fluctuations. The long term

drift of the optical system can be canceled out to an extent by taking the spectra in a sequential way. The experimental system is frequently checked by measurements of toluene as a control, and usually the subtraction error can be suppressed below 0.3% in our set-up as calculated by $|A-B|/|A+B|$, where A is the spectrum taken in one chamber and B is in the other.

ii). **Special Techniques for photoisomerization**

As we mentioned earlier in Chapter 1, a major difficulty we encountered in obtaining the Raman spectra of retinoids is their photolability, i.e. they are isomerized into other isomer configurations upon absorbing a photon, and this will give rise to a Raman spectrum of a mixture of different species. To solve this problem, two methods are usually employed as described below.

a). *Molecular flow technique*

Resonance Raman spectroscopy is a major tool for the studies on the retinal-based pigments. To apply this technique, one needs to make sure that the sample under study is fresh in order to obtain a pure Raman spectrum of the starting photosensitive species. The molecular flow resonance Raman technique and analysis developed earlier in our laboratory (Callender, *et. al*, 1976) can be used on these photolabile samples in solution. It involves imposing a molecular velocity transverse to the Raman exciting laser beam sufficient to ensure that any given molecule moves through the laser beam faster than it has any significant probability of absorbing a photon. In this way, the Raman apparatus detects essentially only the

nonphotoisomerized pure starting material even though absorption is taking place. The photoisomerized molecules are simply removed from the laser beam faster than they can accumulate and are replaced by the original sample. Experimentally, this is accomplished by flowing the material through the laser probe in a capillary tube. This method, however, usually requires quite large amount of sample. Unfortunately, for the time being, our samples are very limited, and it is not feasible for us to apply this molecular flow technique. Therefore we apply another approach to overcome the problem of photosensitivity of retinal chromophores that will be described next.

b). *Pre-resonance Raman Technique.*

Theoretically, when a Raman excitation light coincides with the next electronic level above the ground state, resonance Raman effect takes place; if the energy of the light is not high enough to reach the electronic level, but is close to it, this is usually defined as the preresonance Raman effect. Preresonance Raman spectroscopy is a tradeoff on the scattering intensity for the reduction of photoisomerization of retinal molecules. In this technique, a far-red laser line is utilized to excite Raman scattering on a stationary sample, red shifted far enough from the absorption center of chromophores, insuring no significant photo isomerization but yet retains a resonance effect. Actually, there is a slow replacement of sample in the laser beam due to diffusion. Even though the resonance effect is attenuated, the comparison of the chromophore over that of the protein is still considerable. This method has been

tested on 11-cis retinal ($\lambda_{\max} = 380 \text{ nm}$) with 676 nm line and its protonated Schiff base (PSB) ($\lambda_{\max} = 440 \text{ nm}$) with 752 nm, and no difference was found between our results and those obtained by the flow technique. This approach was also applied by other groups, and no evidence of isomerization was observed (Curry, *et al.*, 1982; Smith, *et al.*, 1985). Furthermore, the amount of sample needed to obtain high quality Raman spectrum is only about 20 to 30 μL . For this reason, we employ the pre-resonance Raman technique to acquire Raman scattering data. In our laboratory, there are several far-red laser lines available from a Krypton ion laser.

iii). Experimental Set-up

The Raman spectra are measured using a Raman difference spectrometer employing an optical multichannel analyzer (OMA) system as depicted in Figure 2.4. In our pre-resonance Raman study, 50 mW of 676 or 752 nm light from a krypton ion laser is used as Raman excitation sources. We can simultaneously detect the Raman spectrum with a 1000 cm^{-1} spectral range. The samples under investigation are loaded into a specially designed 2 or 3 chamber cuvette, which is mounted on a translator stage-stepping motor combination. Each chamber of the cuvette has an inside dimension of $3 \times 2.5 \text{ mm}$ and is loaded with about 30 μL of sample in a typical experiment. Sample concentrations are usually about 1 mM. Typically an experiment takes one to two hours with a signal-to-noise ratio of better than 1000/1. Several precautions must be taken to minimize systematic error. The entire spectrometer system, including the exciting laser, is mounted on a vibration-free table.

Ambient room temperature is controlled to within ± 1.5 °C and relative humidity to within $\pm 3\%$. The protein solution is loaded into one side of the split cell cuvette, (e.g. the A side), while the protein/ligand complex is added into the other (the B side). After measuring the Raman spectra of the two samples sequentially, one then calculates the Raman difference spectrum of the bound substrate from the two spectra stored in the laboratory computer. Because it is very difficult to match the signal level arising from chamber A and B, the difference spectrum that nulls out the protein background signal is $A - \alpha B$, where α is determined by trial subtractions that vary this parameter until the background peaks of the protein, which are generally relatively broad and small under pre-resonance condition compared to those of the retinal chromophore, subtract out.

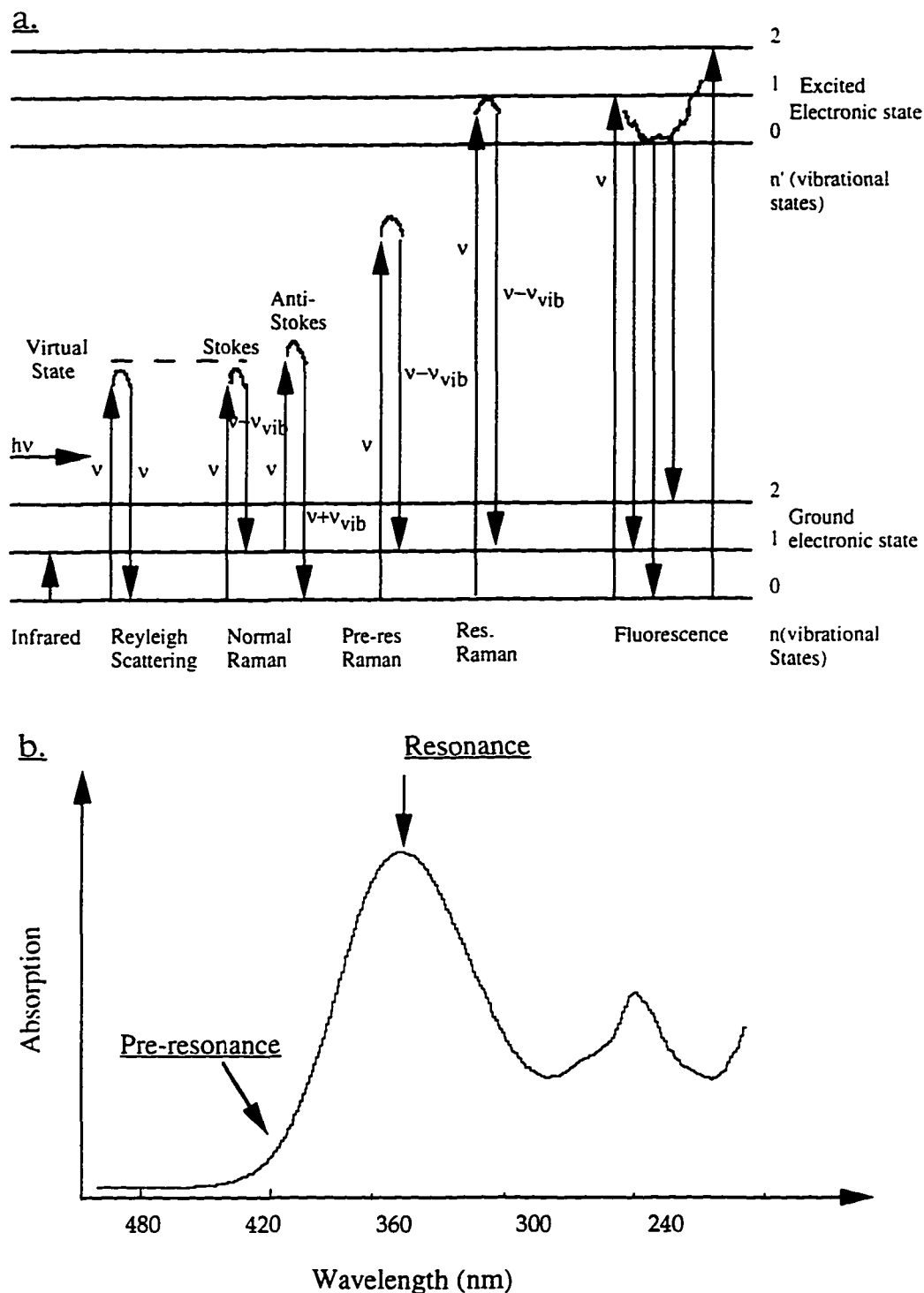


Figure 2.1 (a). The energy diagram of photon-molecule interactions. (b). Areas of pre-resonance and resonance in an absorption band.

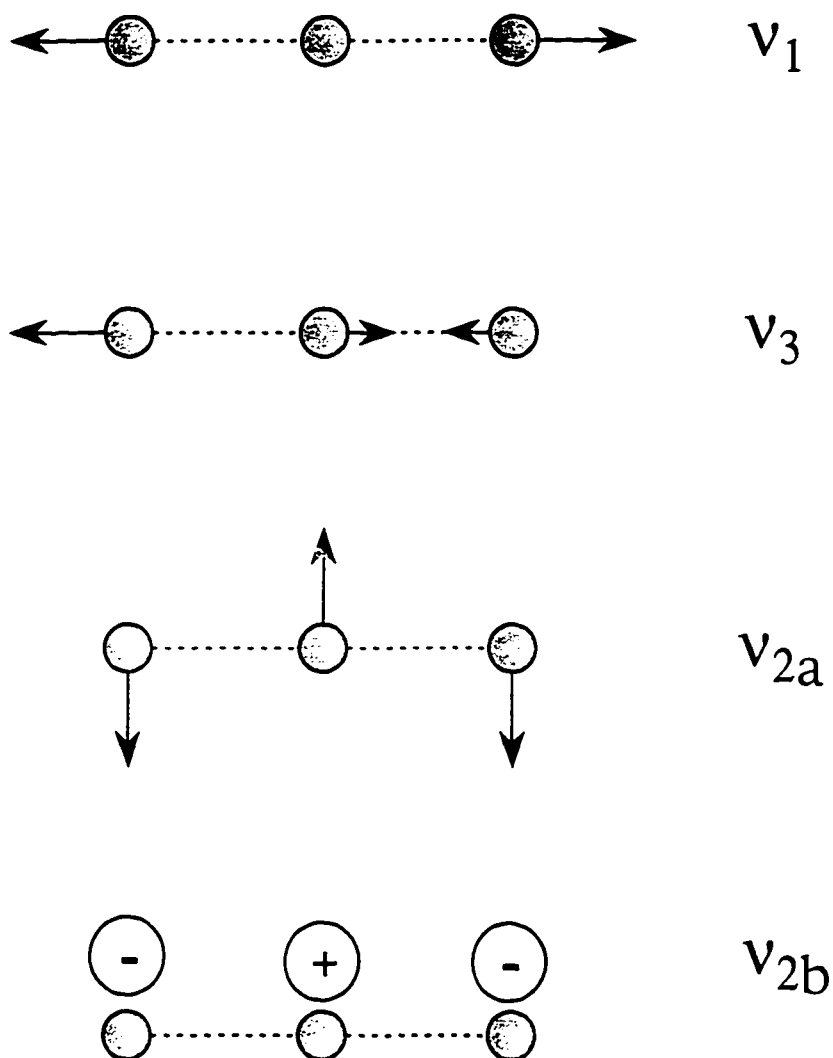


Figure 2.2 The normal modes of carbon dioxide. The nuclei move in the direction of the arrows. In the ν_{2b} mode the $\oplus$ and $\ominus$ signs indicate that the nuclei move perpendicular to the plane of the page toward and away from the reader, respectively.

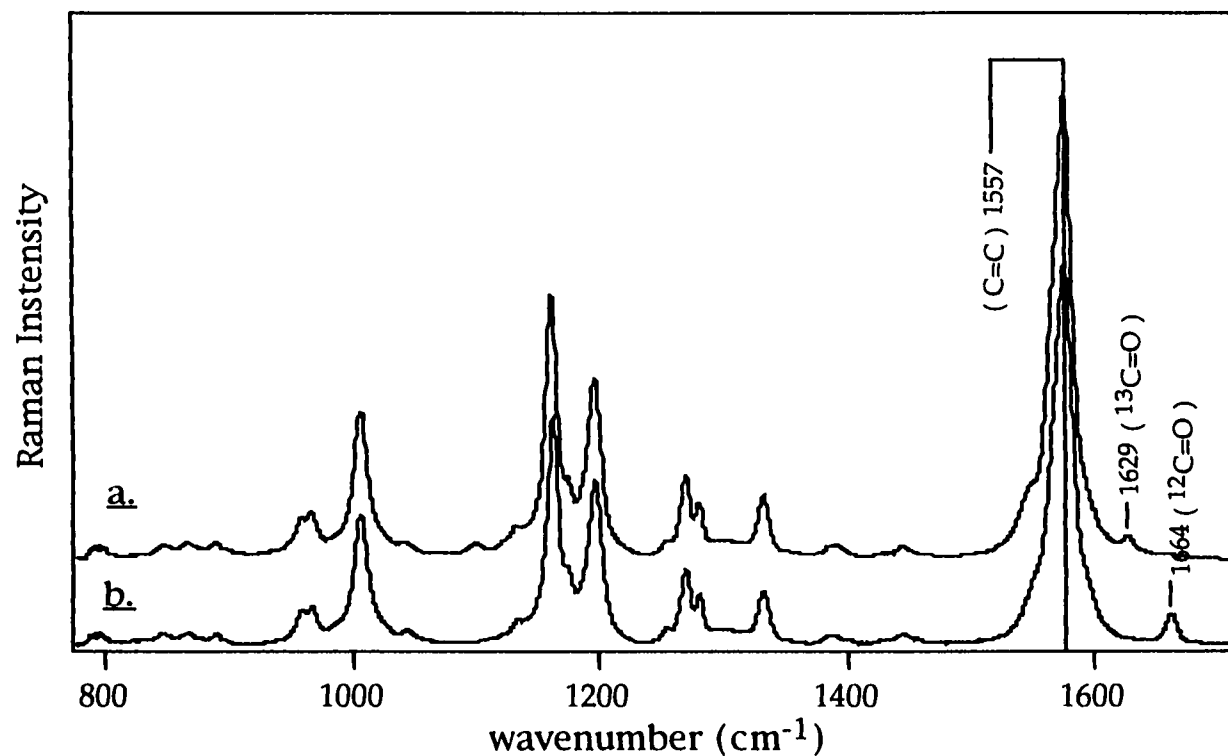


Figure 2.3 Raman spectra of ¹³C labeled at the 15 carbon position (a) and unlabeled (b) all-trans retinal in CCl₄ solution. Sample concentrations are 1 mM; Raman excitation light is 676 nm from Kr⁺ ion laser at 50 mW. The band of 1664 cm⁻¹ is shifted to 1629 cm⁻¹ due to the ¹³C-isotope substitution and the rest bands in the spectrum are not significantly affected as shown in the picture.

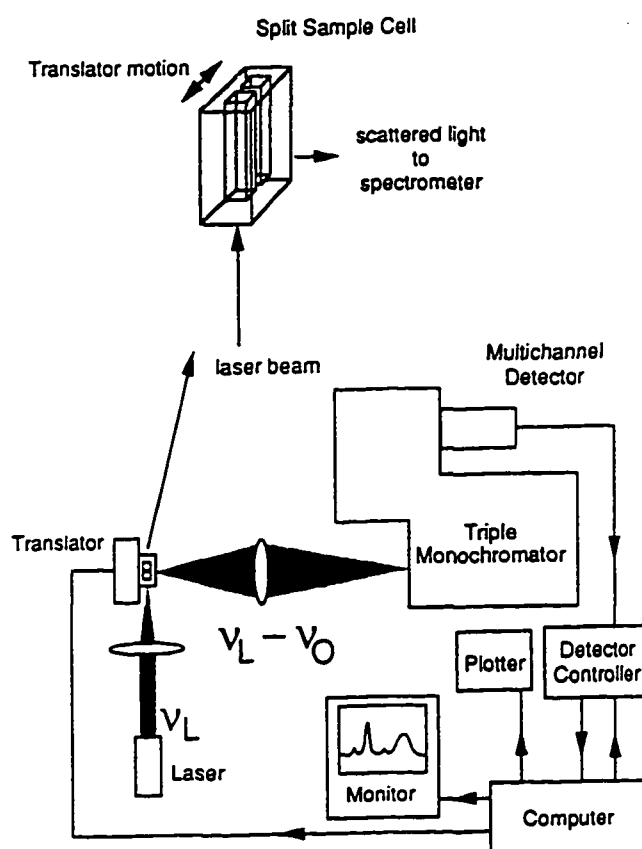


Figure 2.4 A schematic drawing of the instrumental set-up for Raman measurement. The monochromatic light emitted from a laser source is introduced through the sample. The scattered light is focused on the entrance slit of a triple spectrometer. The spectrometer disperses the incident light into a frequency spectrum and focuses it on to a detector. The spectrum was converted into electrical signals by a charge coupled device (CCD), and transferred to a computer for analysis.

Chapter 3

SAMPLE PREPARATION

I. Proteins

Bovine Retinol binding protein (RBP) is purified from bovine serum (Pel-Freeze Co.), utilizing a transthyretin (TTR) affinity gel as described by Vahlquist and co-workers (Vahlquist, *et al.*, 1971); it was found to contain 0.85-1 mol retinol/mol protein by absorption spectroscopy. Transthyretin was purified similarly using an RBP affinity gel. Both proteins were >85% pure by electrophoretic criteria. Apo-RBP is obtained by extracting retinol from bovine RBP into heptane. Equal volumes of a holo-RBP in water and heptane were mixed gently at room temperature for 4 hours. Over 95% of bound retinol was removed by this procedure as judged by the ratio of absorbances at 280 nm and 330 nm. Concentrations of RBP, apo-RBP and of retinoids were determined from their absorption coefficients (Hase, *et al.*, 1976; Horwitz, *et al.*, 1974). To obtain retinoic-acid-RBP or retinal-RBP complexes, the appropriate retinoid was added from a concentrated ethanolic solution to apo-RBP, yielding a final protein/retinoid molar ratio of 1:0.85. After equilibration, the protein solutions were concentrated and ethanol was removed by washing three times in a Centricon centrifugal

concentrator (model 10, Amicon Corp., Beverly, MA) with 200 mM NaCl, 20 mM Hepes pH 7.0. All operations were carried out at 4 °C in the dark or under dim red light.

Cellular Retinaldehyde Binding Protein (CRALBP) was purified from retinal pigment epithelium cells by Dr. John C. Saary at the School of Medicine, Department of Ophthalmology, University of Washington, Seattle (Saary and Bredberg, 1982). 300 mL of preparation of CRALBP complexed with normal and $15\text{-}^{13}\text{C}$ labeled 11-cis-retinaldehyde was sent and used without further treatment. The protein concentration is approximately 14 mg/ml, or 0.39 mM in 11-cis-retinaldehyde. This is based on an extinction coefficient at 425 of $15,300\text{ M}^{-1}\text{cm}^{-1}$. The protein is in 10 mM MOPS, pH 7. Due to the presence of small amount of all-trans-retinaldehyde, the chromophore absorption peak is slightly blue shifted from 425 to 421 nm. Apo-CRALBP is not available, because it is not stable without retinal ligand, checked with the regenerability of holo-protein.

Apo-CRBP-I was purified from *Escherichia coli* which was grown at Dr. Noy Noa's laboratory (Department of Medicine, Cornell University Medical College, Ithaca, NY) following the protocol of early work (Levin, *et al.*, 1984). Purification of this protein after its expression in *Escherichia coli* offers several advantages: (1) large quantity of protein is easily obtained; (2) only one retinoid-binding protein is expressed because *E. coli* does not possess any endogenous retinoid-binding proteins; (3) because *E. coli* does not require retinoids for growth and endogenous retinoids are not present, the

retinoid-binding protein purified from this prokaryote can be obtained as apo-protein.

Purification of *Escherichia coli* expressed CRBP-I has to do through the procedures as described below.

To break the cell, an aliquot of the *E. coli* cell paste (stored under -80 °C freezer) is first thawed to 4 °C and suspended in 2 volumes of lysis buffer [TEDA (20 mM Tris, 1 mM EDTA, 1 mM DTT, 1 mM Sodium Azide, pH 8.0), 0.2 M EDTA, 200 mM PMSF, 0.01 mg/mL Aprotinin, 0.01 mg/mL Leupetin, 0.1 mM Benzamidine]; homogenize to disrupt bacteria; then 100 mg/mL lysozyme dissolved in lysis buffer is added. When the suspension gets gooey, 30 mL lysis buffer is added, 10 mL of 1 M MgCl₂ and 20 mg DNase is added; the solution is well mixed, stirred on ice for 30 min. until no longer viscous (it sometime takes a longer time); 40 mg DOC is added next, which is dissolved in 3 mL TEDA, and 100 mL PMSF; This is homogenized again, and stirred on ice for another 30 minutes. Cellular debris is removed by centrifugation with SS-34 rotor at 5000 g for 1 hour at 4 °C. The solution is "Fortified" with 200 mM PMSF.

Ammonium sulfate fractionation of the supernatant fraction is a very first step in the purification: most bacterial proteins are insoluble in solutions of NH₄SO₄ at 70% saturation, in contrast to the cellular retinol-binding proteins which remain soluble. Thus, the supernatant obtained as described above is adjusted to 70% saturation with NH₄SO₄ containing 15 mM dithiothreitol (titrated with 1 M KOH to pH 7.1) with constant stirring over an hour. The suspension is subjected to centrifugation at 41,000 g for 30 minutes at 4 °C. The supernatants which contain CRBP-I are pooled.

The over 70% NH_4SO_4 supernatant containing CRBP-I is then dialyzed with Cellulose Ester molecularporous dialysis membrane with cutoff of 12,000 - 14,000 Dalton overnight at 4 °C against 20 liters potassium phosphate buffer [20 mM, pH 7.1, EDTA (1 mM), 2-mercaptoethanol (10 mM), sodium azide (0.05%), PMSF (0.02%), pH 7.1]. The concentration of the protein solution is further brought to ~5 mg/mL with an Amicon YM10 membrane before gel-filtration column chromatography.

The dialyzed and concentrated protein solution is then fractionated through Sephadex G-50 equilibrated with the same buffer as the protein solution. The protein solution is pumped into the column, and the column is immediately started at a flow rate of 120 mL/hour in XK-50 column. Column fractions are assayed for protein by measuring the absorbance at 280 nm (see Figure 3.1 of absorbance chart). Selected fractions are then surveyed by electrophoresis through a 20% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS-PAGE). The results obtained after Coomassie blue staining of this gel are shown in the inset of Figure 3.1. The elution volume of each of the fractions is labeled. The position of migration of authentic rat liver CRBP-I is noted by the arrow. M_r markers are indicated at left.

The pooled fractions are applied to a Pharmacia (Piscataway, NJ) Mono Q column equilibrated with imidazole (20 mM, pH 6.8) and sodium azide (0.05%). CRBP-I is eluted with a continuous NaCl gradient (0 mM-100 mM) in the same buffer. Two CRBP-I peaks elute at approximately 60 and 70 mM NaCl (see Figure 3.2). These peaks correspond to CRBP-I without and with its initiator methionine

residue, respectively. The spectrofluorimetric and ligand binding properties of Met^+ and Met^- CRBP appear to be identical (Levin, M. *et al.*, 1988). Raman spectroscopic study showed the same result (data not shown). Therefore, the two eluted fractions were pooled into one. The final solution is dialyzed against storage buffer (see below) for overnight.

Purified *E. coli*-derived rat apo-CRBP-I is stored at 4 °C in phosphate buffer (20 mM, pH 7.4) supplemented with 2-mercaptoethanol (1 mM), EDTA (1 mM), and NaN_3 (0.05%). A reliable measurement of CRBP-I concentration is required for our Raman spectroscopic experiments. The measurement in our studies is determined by the molar extinction coefficient for *E. coli*-derived CRBP-I, which is $28,080 \text{ M}^{-1} \text{ cm}^{-1}$ (Li, *et al.*, 1987). Activity of CRBP-I protein was checked by mixing the protein with all-trans retinal at ratio of 1:9.0. The absorption peak was found at 408 nm about 28 nm shifted from that of 380 nm as observed in CCl_4 solution (see Figure 3.3), indicating the normal viability of CRBP-I.

II. Synthesis of Retinal-Related Model Compounds: $15\text{-}^{13}\text{C}$ Edited Retinals, 11-*cis* Retinal Schiff-Base (SB) and Protonated Schiff-Base (PSB).

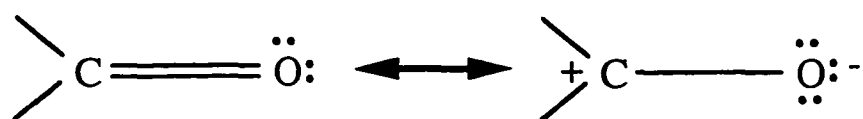
11-*cis* retinal was donated by Dr. Crouch at the Medical School of the University of South Carolina. All-*trans* retinal was purchased from Sigma Chemical Company. The synthesis and characterization of the $15\text{-}^{13}\text{C}$ isotopically substituted derivatives of retinal compounds have been described by Lugtenburg and co-workers (Lugtenburg, 1985). $15\text{-}^{13}\text{C}$ labeled 11-*cis* and all-*trans* retinals are provided by Dr. J. Lugtenburg (Department of Chemistry, University of Leiden,

The Netherlands). The 11-*cis* retinal Schiff base was synthesized by reaction of 1 mg of retinal with a 20-fold excess of n-butylamine in 1 mL of hexane at room temperature for 30 minutes. Subsequently, the solvent and the excess n-butylamine were evaporated under a stream of argon, and the residue was divided into two aliquots, one for Schiff base measurement prepared in CCL₄ solution and the other one for protonated Schiff base preparation. Schiff base formation was assayed by UV absorption spectroscopy which showed that $\lambda_{\max}$ was shifted from about 380 nm as retinal to 360 nm when converted to Schiff base (see Figure 3.4) (Smith, *et al.*, 1985). Since the 11-*cis* retinal protonated Schiff base is unstable in CCl₄ solution (Palings, *et al.*, 1987), the above dried Schiff base was dissolved in 4 mL methanolic solution. Then the solution was titrated with 50 mL of an acidified (12 N HCl) solution of methanol until a stable protonated Schiff base had formed (see Figure 3.4), which was monitored by the absorption spectrum of protonated Schiff base which $\lambda_{\max}$ is positioned at 440 nm upon protonation (Smith, *et al.*, 1985). The presence of excess n-butylamine was found to be essential for the stability of the 11-*cis* PSB. Care must be taken while protonating 11-*cis* Schiff base, because 11-*cis* Schiff base is found to isomerized into all-*trans* Schiff base due to the heat produced by adding concentrate acidic solution in the process of titration. The protonated 11-*cis* retinal Schiff base was carefully checked thereafter through Raman spectroscopy and we found that titration with mixture of 1 mL of 12 N HCl:100 mL of methanol did not cause thermal isomerization.

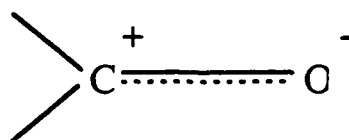
III. ¹⁸O Isotopic Substitution of 11-*cis* Retinal

^{18}O labeled 11-*cis* retinal when complexed with CRALBP will be used to verify the existence of Schiff base or aldehyde based hydrogen bonding linkage between the protein and its retinal ligand. The oxygen atom at the terminal carbonyl $\text{C}=\text{O}$ group of 11-*cis* retinal can be replaced by the oxygen atom in H_2^{18}O due to nucleophilic addition reaction (Solomons, Organic Chemistry, Fifth edition, John Wiley & Sons, Inc., 1992, New York).

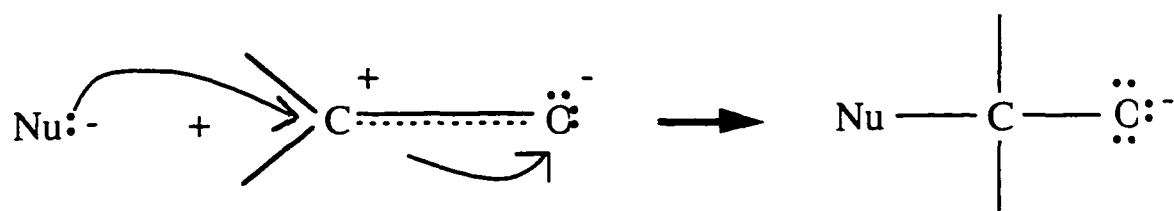
The carbonyl carbon atom is sp^2 hybridized, thus it and the three groups attached to it lie in the same plane. The bond angles between the three attached atoms are what we would expect of a trigonal planar structure, which are approximately 120° (see Figure 3.5). The more electronegative oxygen atom strongly attracts the electrons of both the s bond and the p bond, causing the carbonyl group to be highly polarized; the carbon atom bears a substantial positive charge and the oxygen atom bears a substantial negative charge. Polarization of the p bond can be represented by the following resonance structures for the carbonyl group,



or



The carbonyl group is susceptible to nucleophilic attack because, as we have just seen, the carbonyl carbon bears a partial positive charge. When the nucleophile adds to the carbonyl group it uses its electron pair to form a bond to the carbonyl carbon atom. The carbonyl carbon can accept this electron pair because one pair of electrons of the carbon-oxygen double bond can shift out to the oxygen.



As the reaction takes place, the carbon atom undergoes a change in its geometry and its hybridization state. It goes from a trigonal planar geometry and sp^2 hybridization to a tetrahedral geometry and sp^3 hybridization. For retinal compounds, the reaction is more favored due to the π electron system along the isoprene chain.

Applying the principle discussed above, we successfully performed ^{18}O isotopical substitution on the carbonyl group of 11-*cis* retinal (see Figure 3.6) with H_2^{18}O (cf. Bender, 1951). Because 11-*cis* retinal is not water soluble, we used acetonitrile as medium solvent to dissolve 11-*cis* retinal, as it is capable of accommodating both polar and nonpolar compounds. In our work, 1 mg 11-*cis* retinal was added into 100 fold (mole:mole) H_2^{18}O . Then acetonitrile was added to titrate the emulsive solution until it became clear. The reaction was allowed to proceed overnight at room temperature. All operations were carried out under dim red light or in the dark. The

reaction undergoes the following sequence: the ^{18}O -labeled oxygen with a lone electron pair in water molecule attacks the carbonyl carbon of 11-*cis* retinal, resulting in the change of geometry of the carbon atom from a trigonal planar into a tetrahedral geometry, and a water molecule is linked to the carbon atom; one of the protons attached to ^{18}O atom is transferred to ^{16}O atom; the hydroxyl group with the transferred proton is grabbed by a proton ionized from a water molecule and further detached from the intermediate molecule; on the other side, the hydrogen atom of ^{18}O -H group is pulled away by a water molecule, and an 11-*cis* retinal of ^{18}O isotopically labeled carbonyl group is thus formed. After the reaction, the solution was dried gently under nitrogen flow and stored in a -80° freezer. The reaction product was checked with Raman spectroscopy (see Figure 3.7a). As shown in the picture, about 4/5 of the original 11-*cis* retinal was converted to isotopically substituted molecule without any significant isomerization as compared to the unlabeled one (see Figure 3.7b). The carbonyl stretching band of the isotopically edited 11-*cis* retinal is down shifted from 1666 cm^{-1} to 1638 cm^{-1} as expected.

In principle, the yield of the isotopic substitution should be almost complete because of the overwhelming amount of H_2^{18}O molecule. The less quantity in yield than expected may result from the moisture of air, high percentage of water in the "dry" acetonitrile and short reaction time. An increase of temperature to expedite the reaction is deliberately avoided, because 11-*cis* retinal is subject to isomerization under higher temperature. Fortunately, a 4/5 yield is far enough to give rise to appreciable Raman peak shift.

The reversibility of ^{18}O labeled retinal back to its normal species when bound to protein was investigated with all-*trans* retinal (labeled with the same method as discussed above) complexed with CRBP-I by Raman spectroscopy. No noticeable amount of reversed retinal molecule was observed by comparing the hydrogen bonded $\text{C}=\text{}^{16}\text{O}$ stretching band of both ^{18}O -labeled and unlabeled species at 1645 cm^{-1} (see Figure 3.8). The missing band of $\text{C}=\text{}^{18}\text{O}$ stretching mode in spectrum (a) is down shifted, and coupled with the ethylenic band at 1565 cm^{-1} .

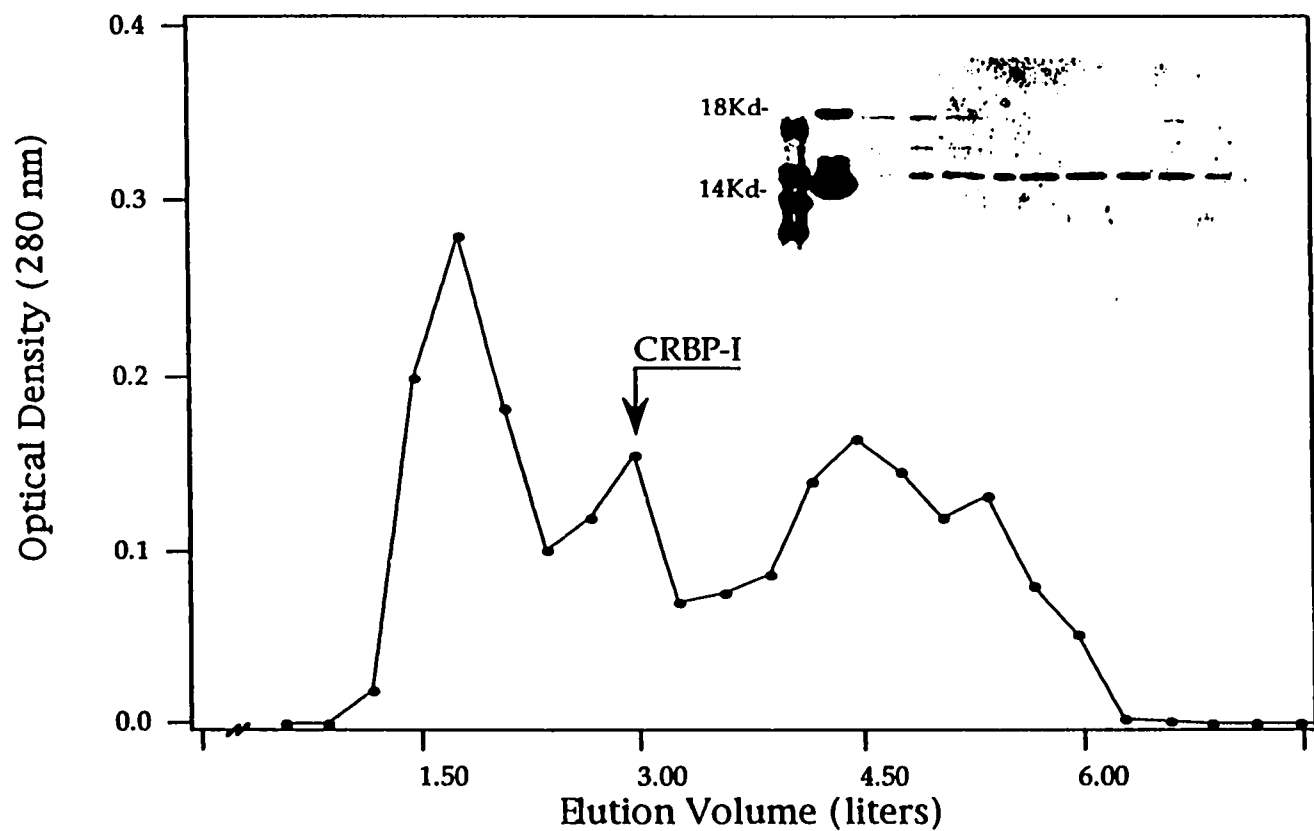


Figure 3.1 Fractionation through a Sephadex G-50 column. Column fractions are assayed for protein by measuring the absorbance at 280 nm. Selected fractions are surveyed by electrophoresis through a 20% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS-PAGE).

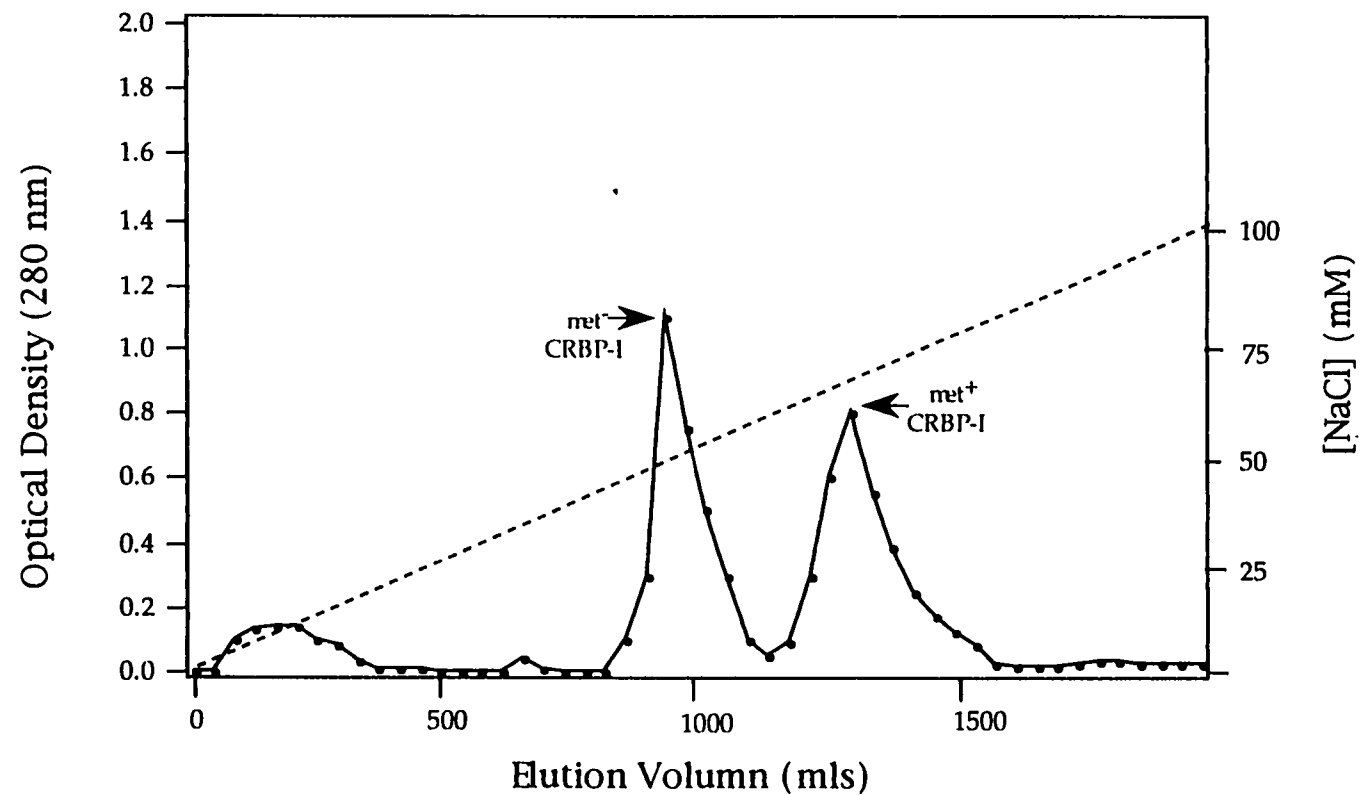


Figure 3.2 Separation of Met⁺ and Met⁻ CRBP-I by fast protein liquid anion-exchange chromatography. Sephadex G-50 fractions containing CRBP-I was loaded into a 30x5 Mono Q column equilibrated with imidazole buffer (20 mM, pH 6.8, plus 0.05% Sodium Azide). The protein solution was fractionated using NaCl gradient shown.

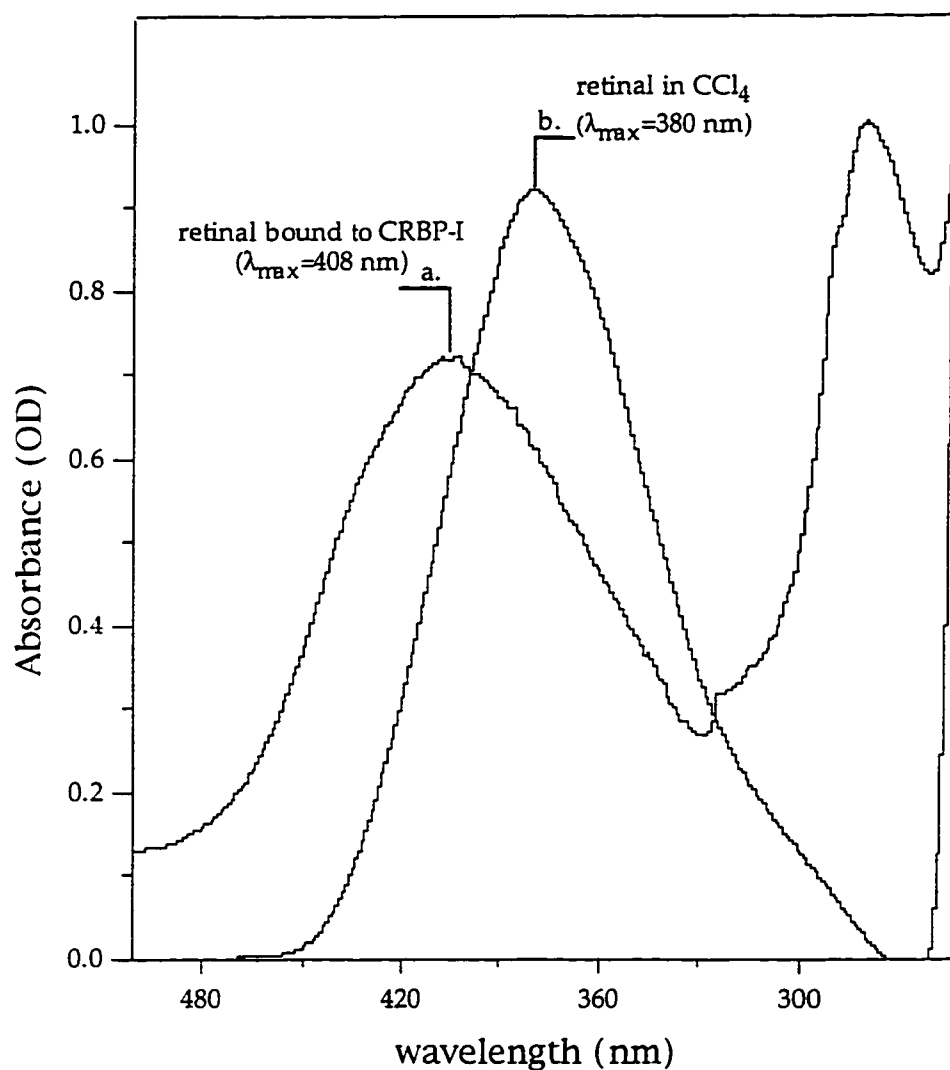


Figure 3.3 Investigation of the activity of CRBP-I. Spectrum (a) is for all-*trans* retinal in CCl_4 solution, which maximum absorption occurs at 380 nm; spectrum (b) is for all-*trans* retinal complexed with CRBP-I with a ratio of 0.9:1.0, and the peak of maximum absorption is at 408 nm, 28 nm shift from that in CCl_4 solution, indicative of the normal functionality of CRBP-I.

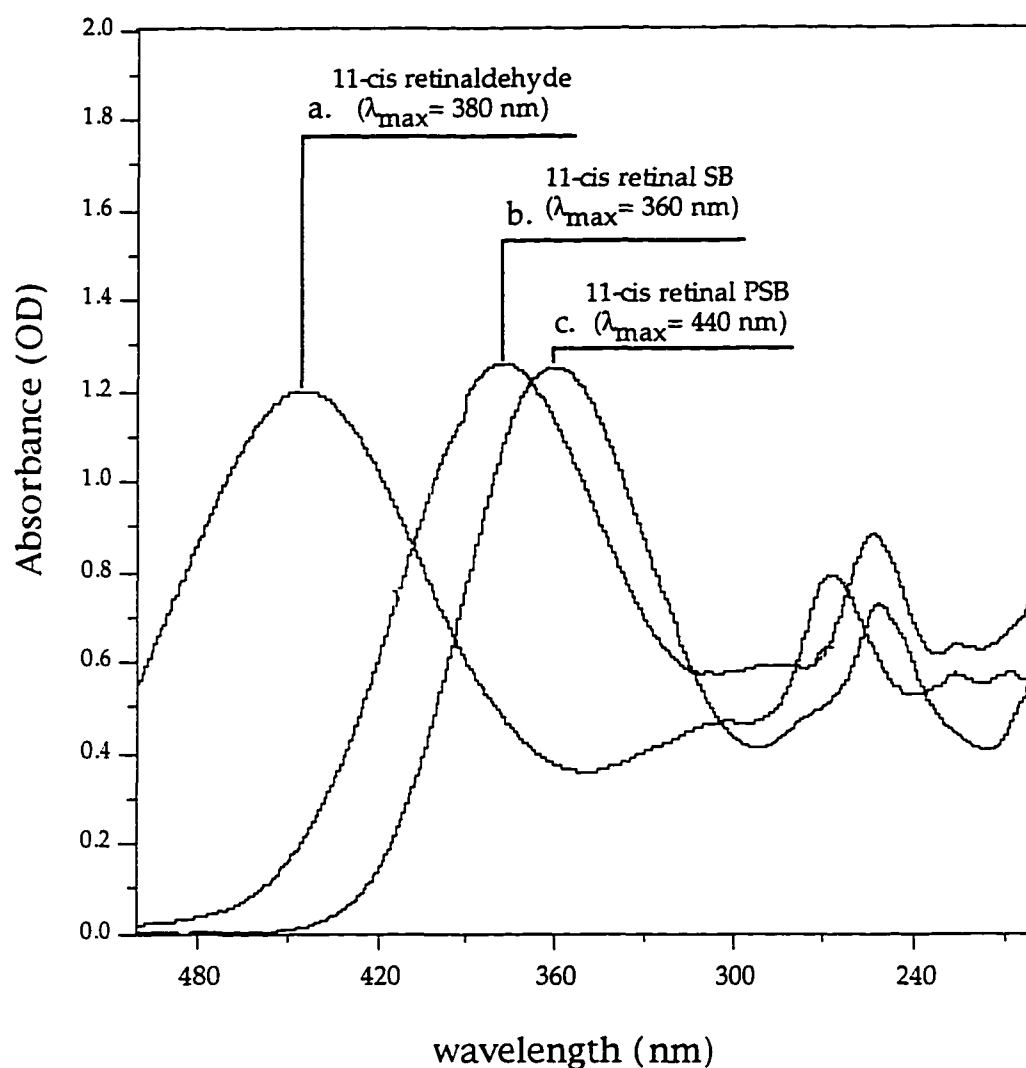


Figure 3.4 Characteristic UV absorption spectra of 11-*cis* retinal and its related Schiff Base and Protonated Schiff base, which are used for the determination of the synthesis of SB and PSB. a). 11-*cis* retinaldehyde ($\lambda_{\max} = 380$ nm); b). Schiff base ($\lambda_{\max} = 360$ nm); c). Protonated Schiff Base ($\lambda_{\max} = 440$ nm).

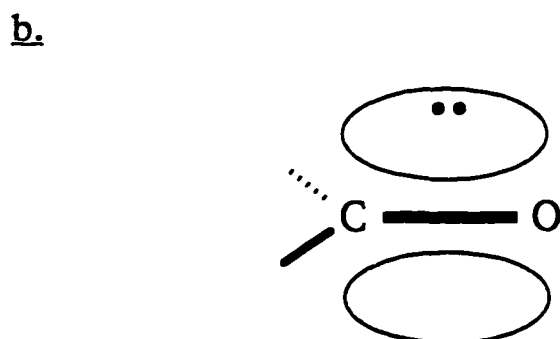
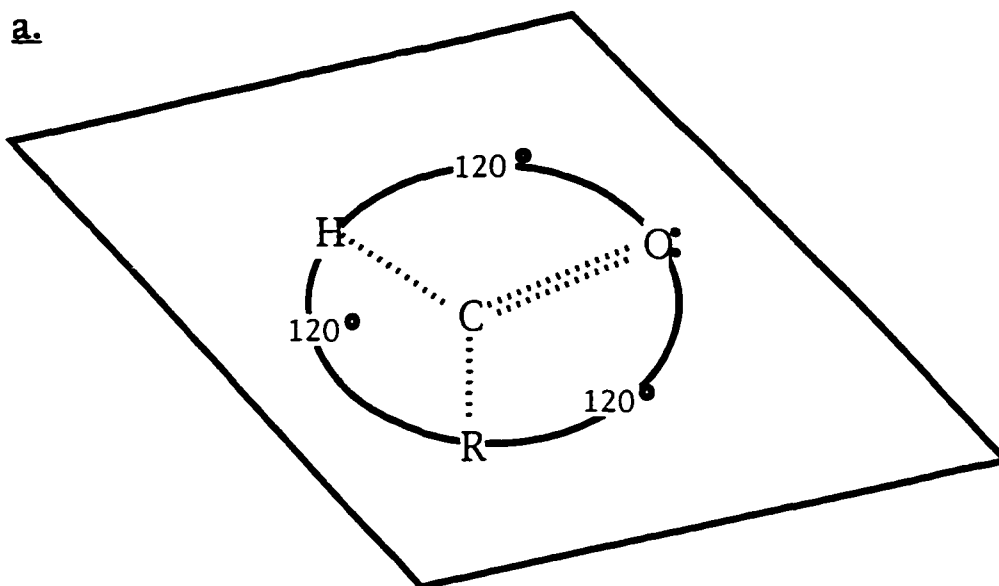


Figure 3.5 (a). Schematic diagram of sp^2 hybridized carbonyl carbon structure. The three groups attached to the central carbon form a plane and the bond angles between the groups are approximately 120 degree. (b). The carbon oxygen double bond consists of a σ bond and a π bond.

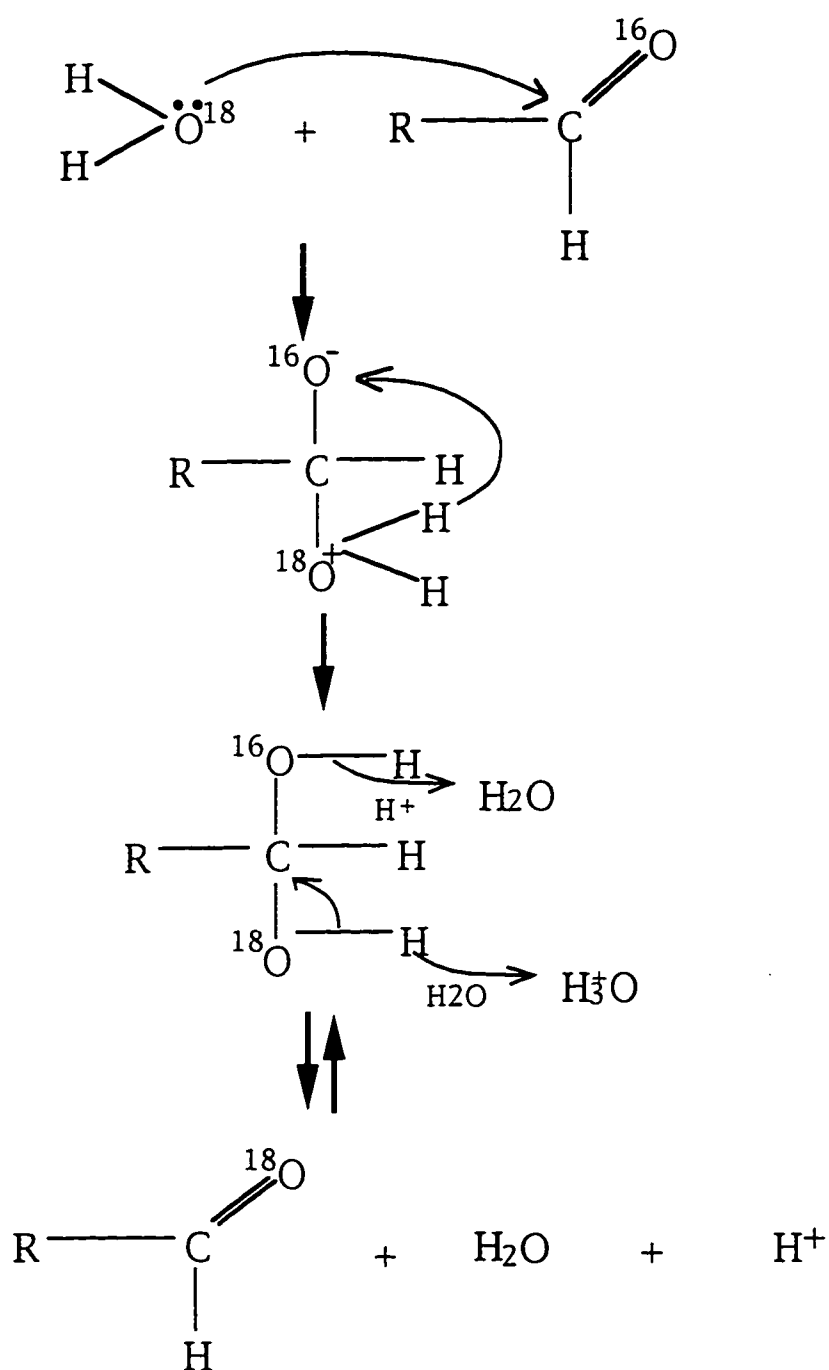


Figure 3.6 Process of ^{18}O isotopic substitution.

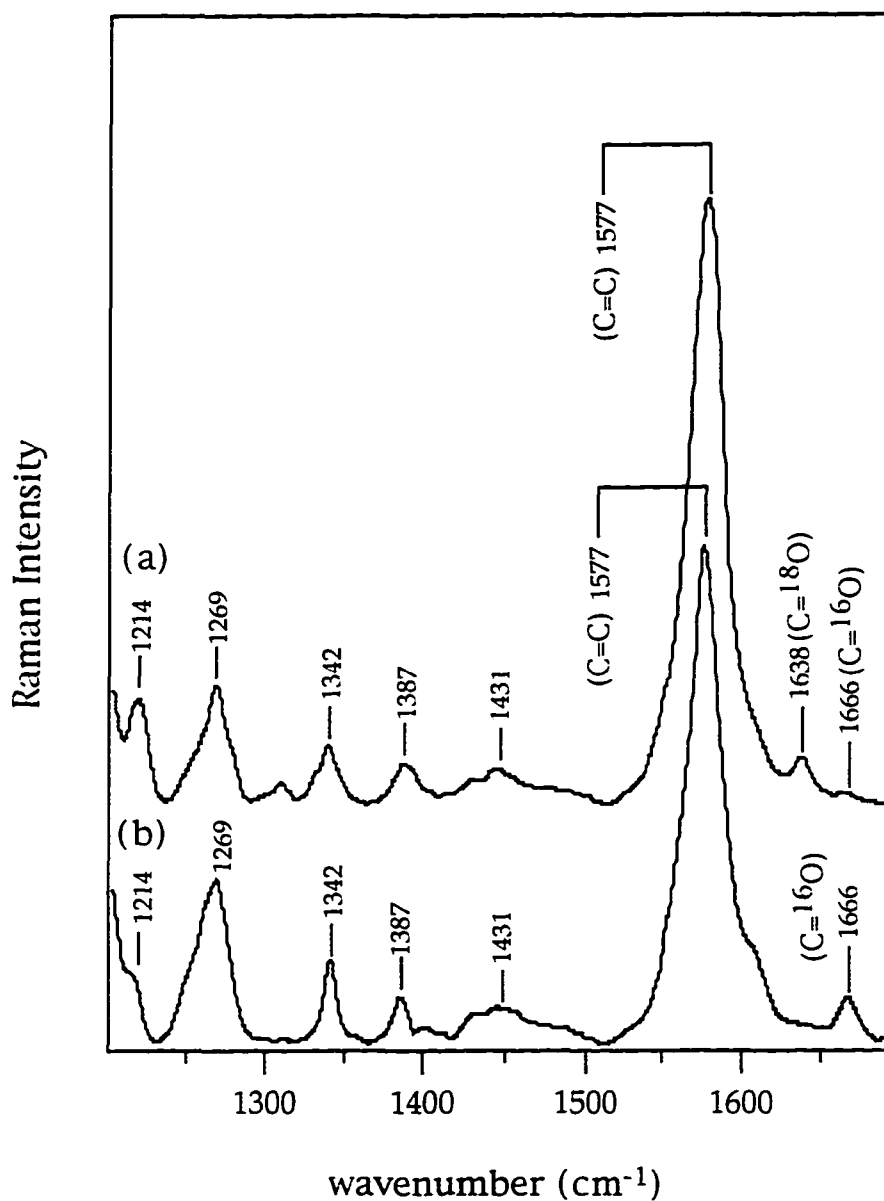


Figure 3.7 Comparison of ¹⁸O labeled 11-*cis* retinal spectrum (a) and the unlabeled (b). The spectra were taken with 750 nm laser line from Krypton ion laser at 50 mW.

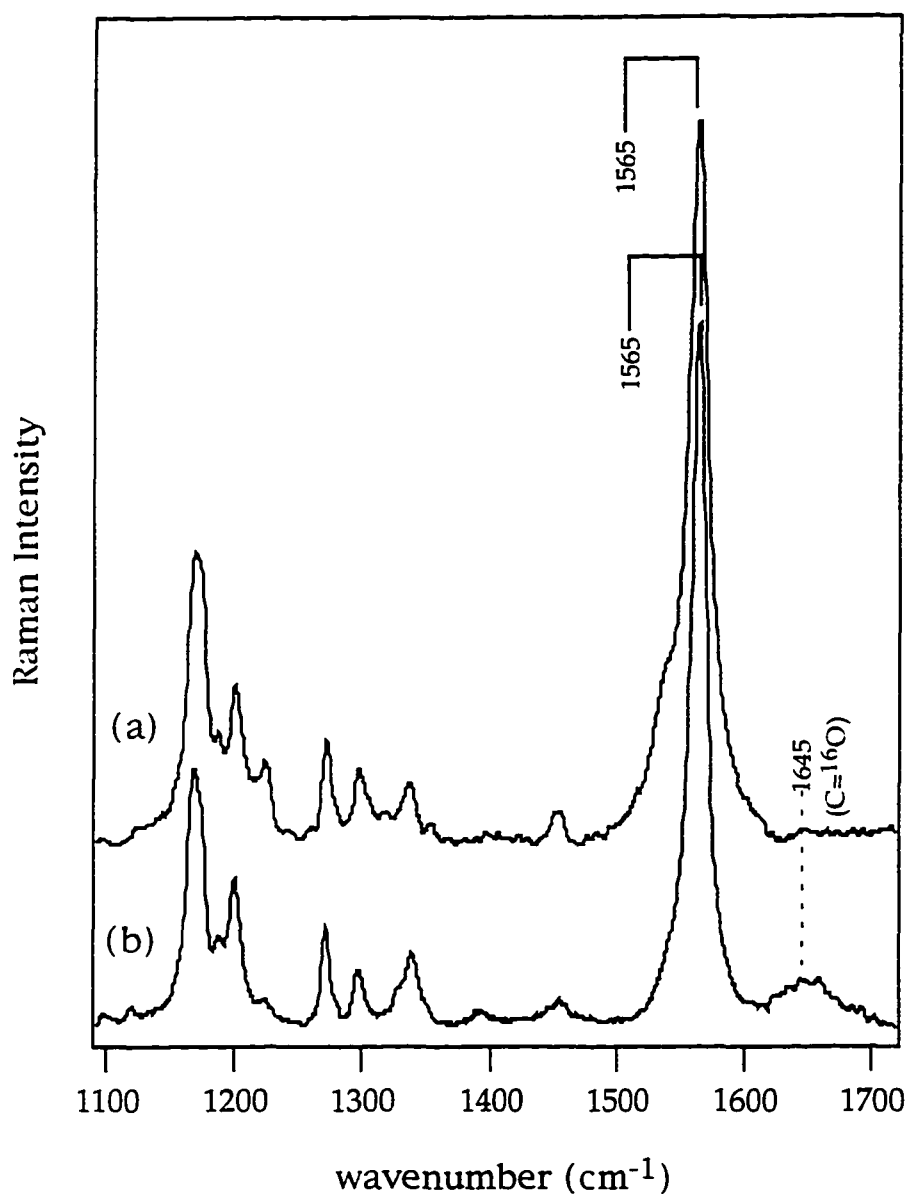


Figure 3.8 Comparison of Raman spectra of ¹⁸O labeled all-*trans* retinal (a) and that of the unlabeled (b) complexed with CRBP-I. 50 mW of 676 nm line from Krypton ion laser was used as Raman excitation source.

Chapter 4

PRE-RESONANCE RAMAN STUDIES OF RETINOL BINDING PROTEIN (RBP)

I. Abstract.

The pre-resonance Raman spectra of retinal, retinoic acid and retinol in dilute CCl_4 solutions and when bound to bovine-serum retinol-binding protein have been measured. The comparison reveals that the binding interaction does not involve any specific interactions of the head group and/or the polyene chain with a particular protein residue. The data indicate hydrogen bonding of bound retinal's head-group oxygen to water, as well as some torsional angle change of its polyene chain upon binding.

II. Introduction

Vitamin A is transported normally in postabsorptive plasma as the lipid alcohol retinol bound to a specific transport protein plasma retinol-binding protein (RBP). The isolation and partial characterization of RBP was first reported in 1968 (Kanai, *et al.*, 1968). RBP is a single polypeptide chain with a molecular weight close to 21,000 Dalton and a single binding site for one molecule of

retinol. In plasma, most of RBP normally circulates as the retinol-RBP complex (holo-RBP). RBP interacts strongly with another protein, plasma transthyretin (TTR), and normally circulates as a 1:1 molar RBP-TTR complex, which affects the interaction between RBP and its retinol ligand. The formation of the RBP-TTR complex serves to reduce the glomerular filtration and renal catabolism of RBP.

The interaction of RBP with TTR is very sensitive to ionic strength, with dissociation of the protein-protein complex occurring at low ionic strength (Peterson, 1971b; van Jaarsveld, *et al.*, 1973a). At ionic strength of 0.002 M or below, there is virtually no complex formation between the two proteins. The interaction of RBP with TTR is also strongly pH-dependent. The maximum binding occurs near physiological pH and falls gradually at lower and higher pH values (van Jaarsveld, *et al.*, 1973a). It was observed that the protein-protein affinity fell rapidly above pH 9 and below pH 5, so that there was no binding present at pH 11.4 and only an extremely small amount at pH 3.5. The interaction of retinol with RBP appears to be stabilized by the formation of the RBP-TTR complex (Goodman, and Raz, 1972; Goodman, and Leslie, 1972). At critical pH environment, where retinol receptors reside, the complex of RBP and TTR is decomposed, thus retinol is released from RBP which is in its molten globule state (Bychkova, *et al.*, 1992) and absorbed by the receptors. This process is not associated with major structural changes in the RBP molecule secondary structure (Rask, 1972).

Vitamin A is mobilized from the liver and delivered to peripheral target tissues as the retinol-RBP complex. Retinol mobilization and delivery are highly regulated processes that are particularly controlled by processes that regulate the rates of synthesis and secretion of RBP by the liver. There is evidence that delivery of retinol to peripheral target tissue may involve specific cell-surface receptors that recognize RBP. The retinol transport system provides an interesting model for the study of protein-protein and protein-retinoid interactions and of characteristics and metabolic regulation of a specific binding and transport system.

The specificity of RBP binding retinoids is very low. Changing the functional end group and the conformation of polyene chain do not significantly affect the binding of the retinoid to RBP. The selectivity of RBP for binding various retinoids and other hydrophobic compounds has been hard to decipher from competition experiments despite intensive efforts. While some groups reported similar affinity of RBP for retinol, citral and β -ionone (Hase, *et al.*, 1976), and interpreted the results to indicate lack of a requirement for a fixed polyene chain length, others (Horwitz, *et al.*, 1974) reported no binding of β -ionone, deducing the opposite conclusion. The isomeric conformation of retinoids was reported to have no effect on binding in some cases (Horwitz, and Heller, 1973) and to completely inhibit binding in others (Siegenthaler, and Saurat, 1987). Similarly, conflicting reports were published regarding the necessity of a six-membered ring structure as well as specific requirement of the

polar group on the isoprene tail (Goodman, and Raz, 1972; Hase. *et al.*, 1976; Horwitz, and Heller, 1974). Thus, no coherent picture exists regarding the specificity of the binding interactions. A recent study of the thermodynamic parameters that contribute to the binding of retinol to RBP have shown that binding is stabilized purely by an increase in entropy and that the enthalpy of binding is approximately zero (Noy, and Xu, 1990). These results were interpreted to indicate that binding is mainly stabilized by hydrophobic interactions, and that the hydroxyl moiety of retinol does not play a role in binding to RBP.

An ideal method for study of chromophores embedded in proteins is resonance Raman spectroscopy. In this form of vibrational spectroscopy, the spectrum of a molecule is selectively probed by choosing an excitation light which overlaps the electronic absorption band of a chromophore. The resonantly enhanced Raman cross section is hence much larger than that of the protein vibrational modes so that the observed Raman spectrum is largely or exclusively that of the chromophore. This permits the study of biological molecules in their physiological environment (Carey, 1982). The information content of a vibrational spectrum is very high. For vitamin A molecules, for which extensive work has been performed with regard to visual pigments and related retinal containing proteins, it is known that the observed spectrum is sensitive to the isomeric form and conformation of the polyene chain, the end group of the chromophore and its environment, and interactions between the chromophore and protein or solution (Spiro, Biological

Applications of Raman Spectroscopy, vol. 2, pp. 1-367, John Wiley & Sons, New York). Thus, resonance Raman spectroscopy is particularly valuable as a non-invasive *in situ* probe of retinol chromophores in retinol-binding proteins.

In this study, initial resonance Raman measurements of the serum retinol-binding protein (RBP) has been carried out. The data show that hydrogen bonding of the polar end group of the retinoid, most likely to water molecules at the protein-solution interface in the binding site, and minor conformational change of the polyene chain result from binding. The data are consistent with the hydrophobic nature of the binding site as determined in previous X-ray crystallographic and biochemical studies.

III. Materials AND Methods

Materials

Chromatography gels were purchased from Pharmacia. All-*trans*-retinol and all-*trans* retinoic acid were from Kodak. All other chemicals were from Sigma Chemical Co. Bovine RBP was purified from bovine serum (Pel-Freeze Co.) utilizing a transthyretin affinity gel as described (Vahlquist, *et al.*, 1971); it was found to contain 0.85-1 mol retinol/mol protein by absorption spectroscopy. Transthyretin was purified similarly using an RBP affinity gel. Both proteins were >85% pure by electrophoretic criteria. Apo-RBP was obtained by extracting retinol from bovine RBP into heptane. Equal volumes of a holo-RBP in water and heptane were mixed gently at room temperature for 4 hours. Over 95% of bound retinol was removed

by this procedure as judged by the ratio of absorbances at 280 nm and 330 nm.

Concentrations of holo-RBP, apo-RBP and of retinoids were determined from their absorption coefficients (Hase, *et al.*, 1976; Horwitz, and Heller, 1974). To obtain retinoic-acid-RBP or retinal-RBP complexes, the appropriate retinoid was added from a concentrated ethanolic solution to apo-RBP, yielding a final protein/retinoid molar ratio of 1:0.85. After equilibration, the protein solutions were concentrated and ethanol was removed by washing three times in a Centricon centrifugal concentrator (model 10, Amicon Corp., Beverly, MA) with 200 mM NaCl, 20 mM Hepes pH 7.0. All operations were carried out at 4 °C in the dark or under dim red light.

Spectroscopy

Raman spectra were measured with a Triplemate spectrometer (Spex Industries, Metuchen, NJ) equipped with a charge-coupled device (CCD) detector (LN/CCD-1152UV with a ST-135 CCD controller, Princeton Instruments Inc., Trenton, NJ) cooled to -100 °C. Data were acquired, stored and analyzed on a Mac-II computer (Apple, Cupertino, CA); 100 mW of the 676.4 nm line from a 3000-CR krypton ion laser or 80 mW of the 488 nm line from an Inova-200-25 argon ion laser (Coherent Laser Group, Palo Alto, CA) was used for Raman excitation of RBP and free retinoids. Using the 676 nm wavelength for excitation does not induce any photo-isomerization which can be monitored at the "fingerprint" region of the spectrum (see below). Spectral lines were calibrated

against known Raman lines of toluene. The peak assignments are accurate to within ± 2 cm^{-1} and the spectral resolution was 6 cm^{-1} .

We have performed these measurements also with RBP-bound retinoic acid. Since the comparison between the CCl_4 solution spectrum and the protein-bound data was essentially identical to the other two derivatives, these data are not shown and are only taken as supporting the conclusion based on retinal and retinol. Hydrogen bonding patterns described here for retinal could not be observed with retinoic acid, since the carbonyl stretching mode of this molecule is too weak to be detected with Raman spectroscopy (data not shown, see Rimai, *et al.*, 1971; Chiara and Waddle, 1980).

IV. Results

The Raman spectra of holo-RBP and apo-RBP are shown in Figure 4.1. The apo-protein spectrum appears similar to other protein spectra, representing contributions from constituent amino acid residues as well as some contribution from backbone secondary structure (e.g. Lord, and Yu, 1970; Otto, *et al.*, 1987). On the other hand, the Raman spectrum of the holo-protein is drastically different. Although the laser excitation wavelength (488 nm) is far from the absorption maximum of the bound chromophore ($\lambda_{\text{max}}=325$ nm) the Raman spectrum of the bound ligand dominates the combined protein-retinol spectrum in Figure 4.1a, as is clear from comparing that spectrum with the apo-protein spectrum. This is a result of the near resonance between the laser frequency and the absorption band of the chromophore.

Since the excitation wavelength is significantly closer to the electronic transition energy of the bound chromophore than to that of the protein, selective enhancement of the chromophore's Raman lines occurs (pre-resonance Raman; cf. Myers, and Mathies, 1987). Thus, the spectra of RBP complexed with a retinoid ligand are dominated by the sharp, pre-resonance, Raman bands of the chromophore. As can be seen in Figure 4.1a, 2b and 4.5b, these spectra also include features arising from the protein, such as the amide I band at about 1670 cm^{-1} or the phenylalanine band at 1003 cm^{-1} . Subtraction of the spectrum of apo-RBP from the spectrum of holo-protein, measured under the same experimental conditions, removes these features and results in a "net" bound retinoid spectrum such as the one shown in Figure 4.2c.

The pre-resonance Raman spectra of all-*trans* retinal and all-*trans* retinol in dilute CCl_4 solution and when bound to RBP are shown in Figures 4.2 and 4.5. Since retinoids are light-sensitive, these spectra were obtained with an excitation frequency in the far red (676.4 nm), which was previously shown to cause no photo-alteration (Callender, *et al.*, 1976; Curry, *et al.*, 1982; and see below). It has been known for many years (Rimai, *et al.*, 1971), that the Raman spectra of vitamin A derivatives are sensitive to the isomeric configuration of the polyene chain and the terminal group. Substantial progress has been made in understanding the vibrational normal modes of these molecules, particularly those of retinal isomers (cf. Curry, *et al.*, 1985). It is convenient and meaningful to group these observed bands into a

number of separate regions that reflect the nature of the normal modes. As the vibrational characteristics of retinal are the best understood of these derivatives, we discuss the spectrum of this molecule in detail and then discuss the spectra of the other derivative.

Ethylenic region

The stretching frequencies of the polyene backbone C=C stretches of vitamin A derivatives and retinal's C=O stretch are found between 1500-1700 cm^{-1} . These modes are generally coupled and delocalized involving many C=C stretch internal coordinates. The main band observed in the spectra of Figure 2 lies at 1577 cm^{-1} . This band is most intense because the normal mode (C11=C12 and C7=C8 stretches combines with various C-C stretches and C-H bending motions; Curry, *et al.*, 1982) modulates the π electrons of the polyene backbone more than any other mode; this results in a greater pre-resonance Raman intensity comparatively as retinal's 380 nm absorption band is a π -to- π^* transition. The position of this band is linearly correlated to the λ_{max} of retinal's absorption band, so that a shift to lower frequency represents a shift to a higher λ_{max} (cf. Kakitani, *et al.*, 1983). No shift is observed between the spectra of retinal and retinol spectrum (Figure 4.2 and 4.5), indicating that the λ_{max} of retinal and retinol is the same in both cases. This result is in agreement with separate absorption experiments (data not shown).

The band at 1663 cm^{-1} observed in the spectrum of retinal in CCl_4 (Figure 4.2a) is assigned to the carbonyl, $\text{C}=\text{O}$ stretching mode of retinal. This band shifts to 1650 cm^{-1} upon binding and is considerably broadened (Figure 4.3). Both the shift and the broadening can almost certainly be understood in terms of hydrogen bonding between the carbonyl group and its environment. The effect of proton donors near the $\text{C}=\text{O}$ bond is to draw electrons from the bond towards oxygen. This polarizes the bond, decreasing the bond order and lowering $\text{C}=\text{O}$ stretching frequency. The effect can be observed in a titration of retinal in CCl_4 with the hydrogen-bonding donor phenol, as is shown in Figure 5.4. Upon raising the concentration of phenol, the hydrogen-bonded fraction of retinal, showing as a broadened peak at about 1650 cm^{-1} , is increased at expense of the "free" fraction. The frequency shift of the $\text{C}=\text{O}$ stretch can be related to the strength of the hydrogen bond (Latajka, and Scheiner, 1990; Thijs, and Zeegers-Huyskens, 1984). Thus the lower frequency of retinal's $\text{C}=\text{O}$ stretch in RBP compared to in CCl_4 , where no hydrogen bond is present, indicates that the carbonyl moiety of retinal forms a hydrogen bond when complexed with RBP. The enthalpy ΔH of this local interaction is determined to be -5.6 kcal/mol by our Badger-Bauer relationship studies

Fingerprint region

A second main vibrational region can be observed in the spectra of Figures 4.2 and 4.5 located between about $1000\text{-}1450\text{ cm}^{-1}$. Vibrational contributions to this region have been assigned,

mostly, to C-H in plane bending modes and C-C stretching motions arising both from intra-polyene chain carbons and C-CH₃ vibrations. This region has been called the "fingerprint" region because of its marked sensitivity to polyene isomer configuration. The spectral pattern is quite different for the all-*trans*, 13-*cis*, 11-*cis* and 9-*cis* retinal isomers (cf. Curry, *et al.*, 1985). All peak positions and intensity ratios of retinal in CCl₄ in the fingerprint region (Figure 4.2a) are essentially identical to those reported earlier in rapid flow measurements (Callender, *et al.* 1976). This shows that our excitation conditions (λ_{max} 676.4 nm) do not induce any significant photo-isomerization reactions under our experimental conditions. Measurements using excitation at lower wavelengths ($\lambda_{\text{laser}} < 568$ nm) resulted in a fairly rapid change in the fingerprint region for both the free-solution and protein bound retinals; at these wavelengths the absorption cross section and/or photo-isomerization quantum yield are sufficiently large to produce other retinal isomers. The observation that there is no change in the fingerprint bands upon binding of retinal to RBP indicates that there is no change in the chromophore's isomeric form (all-*trans*) upon binding. Experiments employing lower excitation wavelengths on the holo protein also showed rapid changes in the fingerprint bands, as those observed for retinal in solution, indicating that the bound chromophore is able to photo-isomerize relatively easily within the binding pocket. This suggests that the binding pocket does not have a particular preference for the all-*trans* isomer.

Hydrogen out-of-plane region

A third identified region in the Raman spectra of vitamin A derivatives lies between approximately 800-1000 cm^{-1} and is called the HOOP (hydrogen-out-of-plane) region. These bands have been shown to arise from out-of plane bending modes of all-*trans* ethylenic hydrogens (Curry, *et al.*, 1982). The bands at 961 cm^{-1} and 968 cm^{-1} in the solution spectra of retinal and retinol data are HOOP modes, assigned to the wagging motion of the hydrogens trans to the C7=C8 and C11=C12 double bonds, respectively (Curry, *et al.*, 1982). These bands are of interest because the intensity and number of bands observed in the region are an indication of departures from planarity with respect to torsional rotations along the chromophore's polyene chain. That the spectral intensity of these modes in the bound data is stronger than that observed in the solution spectra indicates that retinal in the binding pocket is somewhat torsionally distorted by the protein near these coordinates. The intensity of the particular HOOP mode, relative to some internal reference such as the C=C stretching mode intensity, was previously used to quantitate the torsional angle for this particular hydrogen (Eyring, *et al.*, 1982). Although such an analysis requires specific resonance conditions as well as decoupling of the particular mode of interest from others (e.g. by isotopic labeling), we can qualitatively state that there is a change in the torsional angle at either the 7-8 or the 11-12 position.

The conclusions drawn above from the comparison between the spectra obtained for retinal in CCl_4 and when bound to RBP

seem to hold true also for retinol (see Figure 4.5) and retinoic acid (data not shown). Additionally, no changes in the ethylenic or the main fingerprint bands of retinol were observed when holo-RBP was bound to its physiological carrier, transthyretin (data not shown).

V. Discussion

The results of the present study show that the pre-resonance Raman spectra of the three retinoids with different end groups (retinol, retinal and retinoic acid) when free in CCl_4 solution, a solvent which is hydrophobic in nature, are very similar to their spectra when bound to RBP. The results thus indicate that the environment of retinol in the binding site is a strongly hydrophobic one, which is in agreement with both the dominant presence of apolar amino acid residues lining the binding pocket, and the almost complete absence of water in holo-RBP's binding site as seen in the X-ray diffraction data (Cowan, *et al.*, 1990). Since the resonance Raman spectra are sensitive indicators for interactions involving the polar end group as well as the isoprene chain of the retinoids, the results clearly indicate that binding of retinoids to RBP does not involve specific ligand-protein interactions in these regions. These results are also in accord with the reports on the low specificity of the binding to RBP for the end group of retinoids (Siegenthaler, and Saurat, 1987).

Two main differences are found between the Raman spectra of free retinal in CCl_4 solution compared to RBP-bound. One is the

down -shift of retinal's carbonyl stretch, from 1663 cm^{-1} to 1650 cm^{-1} , and the broadening of the bandwidth, indicating hydrogen bonding of the carbonyl to its environment when bound to RBP. Some evidence suggests that this effect results from interactions with water molecules rather than with a protein residue. First, the immediate surrounding of the end group as seen in X-ray crystallographic studies does not contain hydrogen-bonding donor residues. Secondly, retinol's hydroxyl group is seen in X-ray pictures located at the very opening of the binding pocket (Cowan, *et al.*, 1990), and was shown to be susceptible to soluble reducing agents and ions (Horwitz, and Heller, 1973). Thus, we attribute the observed down-shift in the carbonyl stretching frequency to the interaction with water. From the Badger-Bauer relationship studies, the 13 cm^{-1} redshift of the carbonyl band $\text{C}=\text{O}$ is corresponding to -5.6 kcal/mol as a local interaction enthalpy. Retinol may be interacting with the structural water molecule observed in the X-ray measurements as being only 0.27 nm away from retinol's hydroxyl in holo-RBP. Alternatively, retinol may interact with water in the bulk phase. In support of this conclusion, essentially the same effect on retinal's $\text{C}=\text{O}$ band, a downward shift and broadening, was previously observed upon addition of water to all-*trans* retinal in acetonitrile (Allan, and Cooper, 1980; Pande, *et al.*, 1982). The observed broadening of this band upon binding reflects a heterogeneous hydrogen-bonding pattern, resulting in a heterogeneous distribution of carbonyl stretching frequencies.

The second change observed in the pre-resonance Raman spectrum of retinal upon binding to RBP is the increased intensity of the 968 cm^{-1} HOOP band. Clearly, binding of the retinoid into the binding pocket induces some torsion around either the 7-8 or the 11-12 positions of the polyene chain.

These considerations lead us to the conclusion that there are no specific interactions between bound retinoids and any amino acid residues of RBP. Binding, therefore, must be stabilized by other forces. It was previously reported that the binding of retinoids to RBP is stabilized predominantly by hydrophobic forces and that the enthalpy of binding is approximately zero (Noy, and Xu, 1990). These results, taken together with the data in the present study showing that binding of retinal in RBP is accompanied by hydrogen bonding, suggest that the energy of the hydrogen bond of the bound ligand is very similar to hydrogen bonding of retinol in the aqueous phase. In our recent studies on the relationship of $\nu_{\text{C=O}}$ to ΔG_{298} (see figure 5.4b), a ΔG_{298} value of -1.7 kcal/mol was obtained for the putative retinal C=O bonding to the water molecule. This value may be compared to the overall ΔG_{298} of binding value of -8.8 kcal/mol between all-*trans* retinal and RBP, which is obtained from the K_d of the complex. This hydrogen bonding interaction between the carbonyl terminal group and the water molecule only contributes about 20% of the overall binding energy of the protein ligand complex. Binding, thus, results in a very small net enthalpy change. We, therefore, suggest that the energy for binding of retinol to RBP originates from displacement of ordered water molecules that occupy the

binding site in the apo-protein. This hypothesis awaits elucidation of the three-dimensional structure of the apo-protein. However, it is interesting to note in this regard that in fatty-acid-binding protein, a different protein which are observed in the X-ray structure of the apo-protein-binding site are displaced upon ligand binding (Sacchettini, *et al.*, 1989).

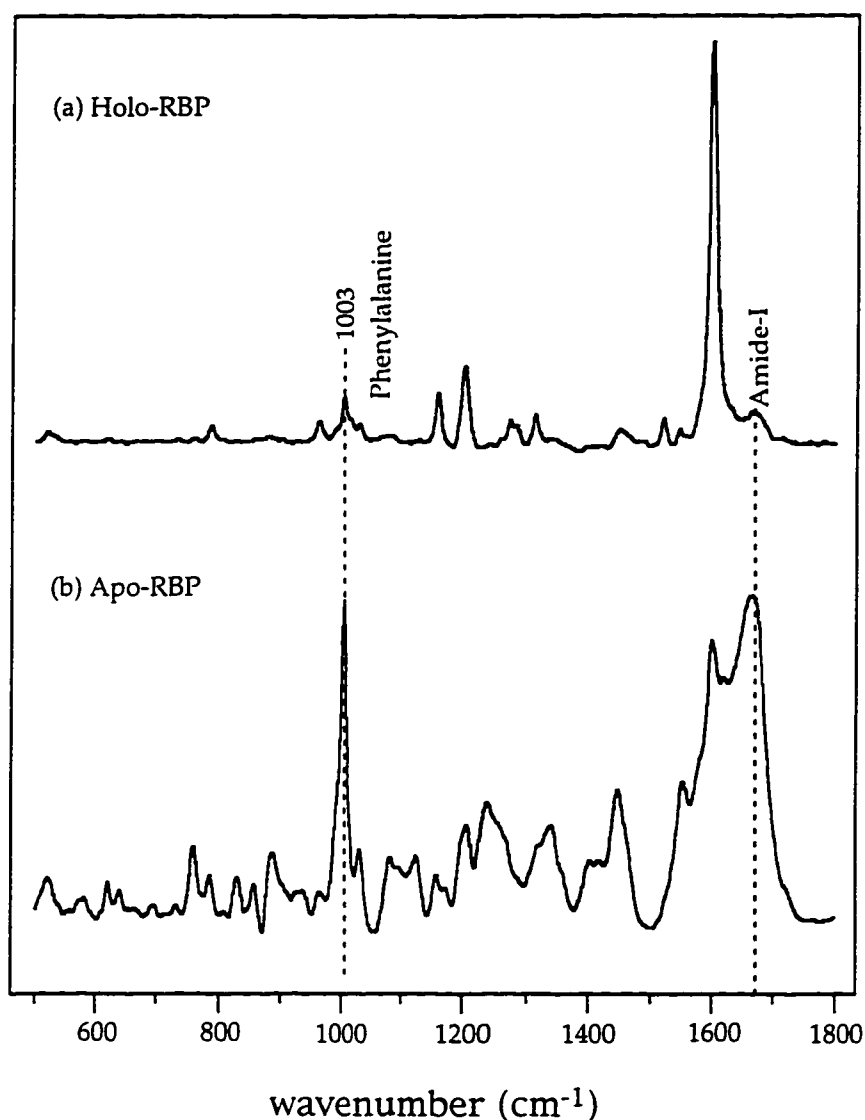


Figure 4.1 Raman spectra of holo-RBP (a) and apo-RBP (b). Protein samples were 0.3 mM (holo-RBP) and 1.5 mM (apo-RBP) in Hepes buffer, pH 7.0. The 488.0 nm line from an Ar⁺ laser was used for excitation at a power 80 mW. Dashed lines indicate Raman bands originating from classical (non-resonance) contribution of the apo-protein.

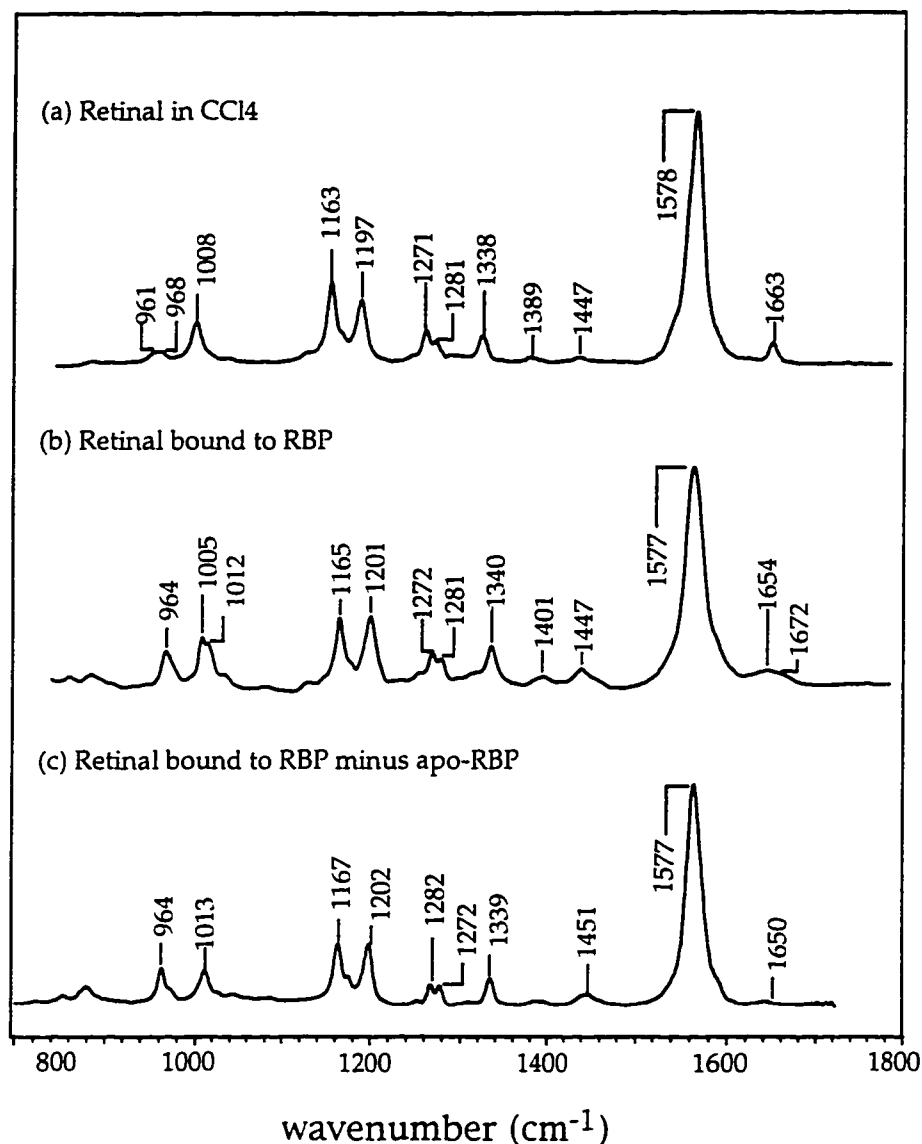


Figure 4.2 Resonance Raman spectra of retinal in dilute solution and when bound to RBP. (a) All-*trans* retinal dissolved in CCl_4 to 0.12 mM; (b) RBP-retinal complex at 0.2 mM in HEPES buffer, pH7; (c) same as (b), after removal of protein contribution to the spectrum by subtraction of an apo-protein spectrum measured under identical conditions. 100 mW of the 676.4 nm line from a Kr^+ laser was used for excitation.

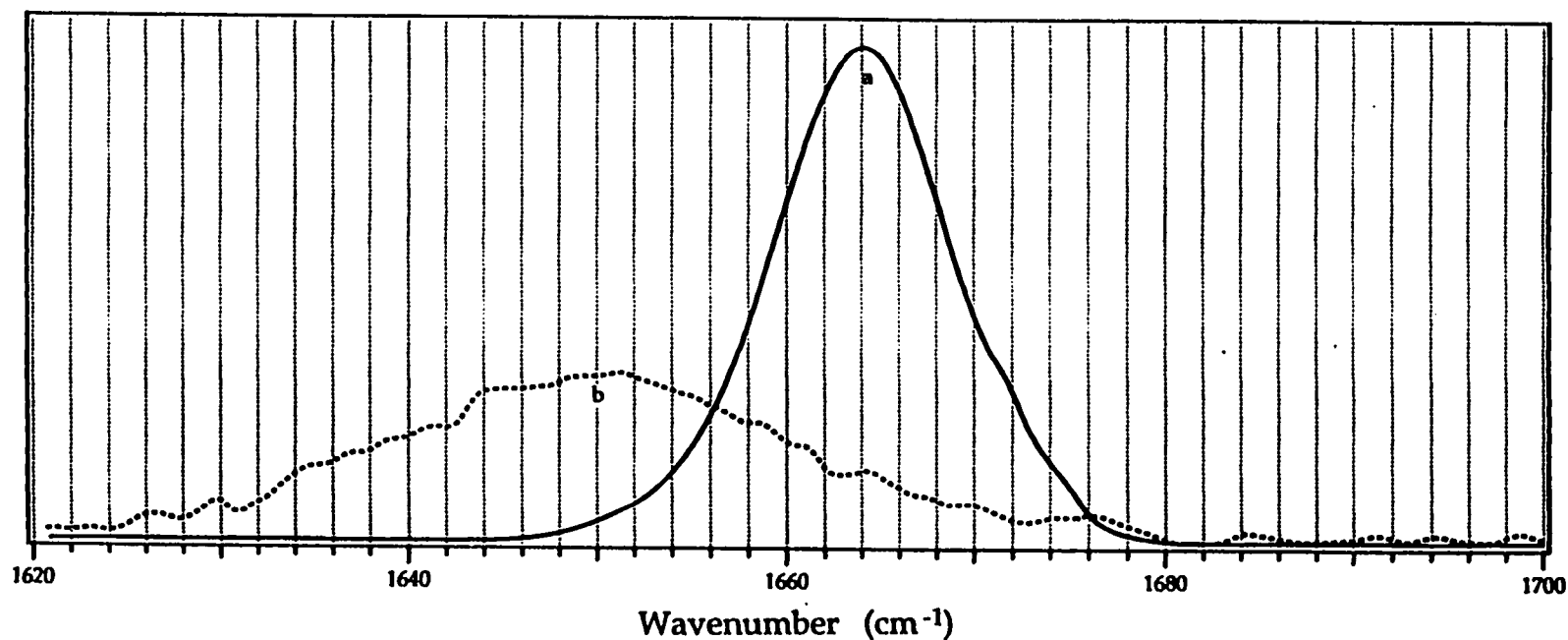


Figure 4.3 Retinal's carbonyl stretching mode when dissolved in CCl_4 solution (a) and when bound to RBP (b). Data are taken from Figure 4.2 and are reproduced here on an expanded scale. In both spectra the intensities are normalized to ethylenic (C=C) stretching mode.

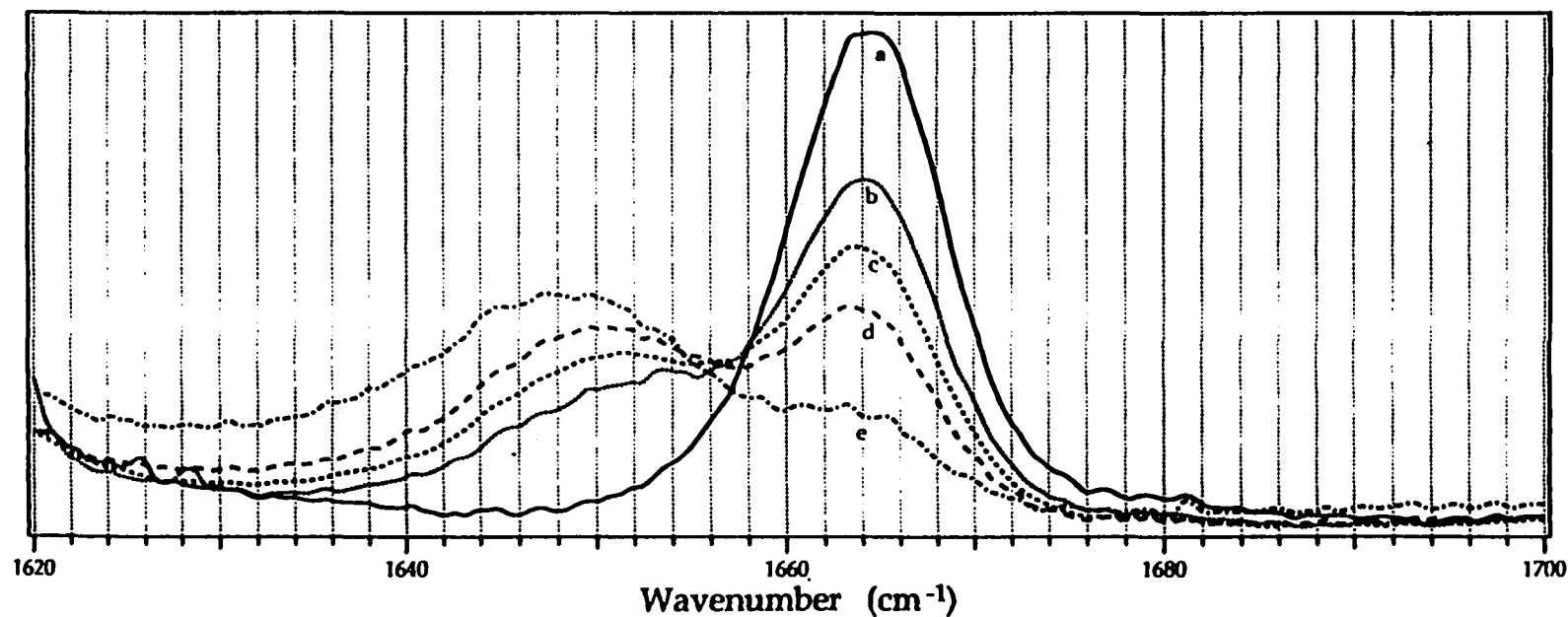


Figure 4.4 Titration of retinal with phenol. Retinal (0.91 mM in CCl_4) was incubated with the different concentrations of phenol. Excitation conditions as in Figure 4.2; temperature 19 °C. Intensities are normalized to the ethylenic (C=C) stretching mode. Phenol concentrations: (a) 0 mM, (b) 18.8 mM, (c) 37.6 mM, (d) 75.2 mM, (e) 150.5 mM.

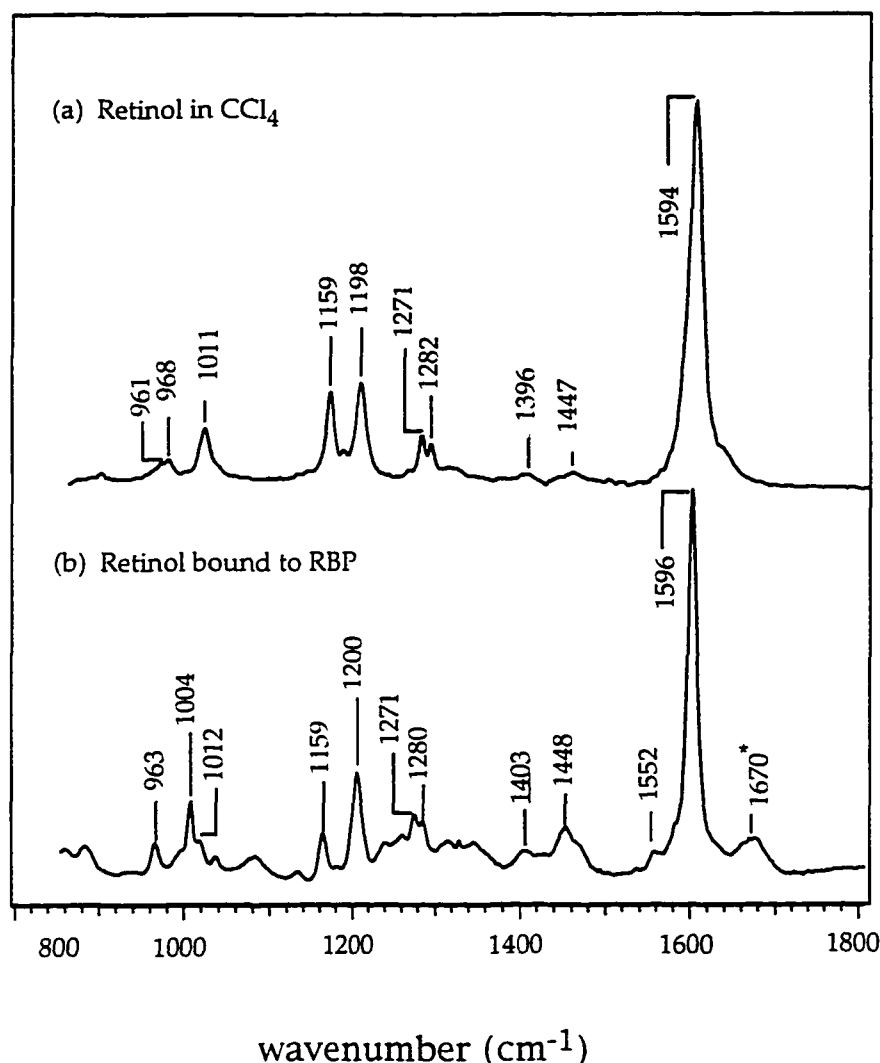


Figure 4.5 Resonance Raman spectra of retinol in dilute solution and when bound to RBP. (a) All-*trans* retinol was dissolved in CCl_4 to 0.13 mM; (b) holo-RBP is at 0.2 mM in Hepes buffer, pH 7.0. Excitation conditions as in Figure 4.2. Note that the holo-RBP spectrum shown here is not a difference spectrum such as shown in Figure 2c. Accordingly, some weak Raman intensities observed here (marked with an asterisk) originate from protein contributions, as seen in Figure 5.2b.

Chapter 5

THE INTERACTIONS BETWEEN CELLULAR RETINOL BINDING PROTEIN (CRBP-I) AND RETINAL: A VIBRATIONAL SPECTROSCOPIC STUDY

I. Abstract

Pre-resonance Raman difference spectra have been obtained for all-*trans* retinal in dilute CCl₄ solution, complexed with Cellular Retinol Binding Protein (CRBP-I), and Retinol Binding Protein (RBP). These spectra indicate that retinal is of a slightly more planar conformation within the binding pocket of CRBP-I than in solution or hydrophobically complexed with RBP. The retinal isoprene tail's conjugated π electrons are perturbed for carbon's from the C8 through C11 positions as compared to retinal in solution or bound to RBP. This perturbation is probably due to the close proximity of the Lys40 in the CRBP-I binding pocket to the above mentioned carbons. The C=O stretching vibration of bound retinal carbonyl has been found to shift from 1664 cm⁻¹ solubilized in CCl₄ to 1650 and 1645 cm⁻¹ in RBP and CRBP-I respectively, and significantly broadened in both cases. The frequency shift and broadening have been attributed to hydrogen bonding. These have been compared to calibrations of frequency shift ($\Delta\nu_{C=O}$) vs. ΔH and ΔG of all-*trans*

retinal complexed with a series of phenol derivatives of incremental proton donating ability as obtained by the relationship of van't Hoff. By this relationship, the binding enthalpy of the all-*trans* retinal carbonyl moiety bound to CRBP-I and RBP is -28.1 KJ/mol (-6.7 Kcal/mol) and -23.5 KJ/mol (-5.6 Kcal/mol), respectively. The free energy of binding of the retinal carbonyl bound to CRBP-I and RBP has been determined to be -10.5 KJ/mol (-2.5 Kcal/mol) and -7.2 KJ/mol (-1.7 Kcal/mol), respectively. The hydrogen bonded C=O moiety of retinal complexed with CRBP-I accounts for a substantial (25%) but not overriding amount of the energy of CRBP-I for retinal and the protein's preference for binding retinal and it also accounts for the protein's preference for binding retinal.

II. Introduction

The importance of retinoid-binding proteins in the physiological trafficking of vitamin A metabolites is well known. There are two distinct families of proteins that have been isolated from various tissues and capable of binding retinoids with high affinity. One family consists of highly homologous proteins. Amongst these are cellular retinol binding proteins (CRBP-I and II), P2 myellin protein, adipocyte lipid binding protein (ALBP), and fatty acids binding protein (FABP). The second family includes serum retinol binding protein (RBP), β -lactoglobulin, and biliverdin binding proteins exhibits lower sequence homology but similar folding topology. The structures of many of these proteins have been elucidated by X-ray crystallography (Cowan, *et al.*, 1990; Newcomer, *et al.*, 1984; Winter, *et al.*, 1993; Xu, *et al.*, 1993; Cowan, *et al.*, 1993). In spite of

similarities in structure, differences in binding specificities between these families indicate differences in the motifs of their ligand binding.

Indicative of the differences between these families, two proteins, retinol-binding protein (RBP) and cellular retinol binding protein (CRBP-I), provide an interesting contrast in their binding of retinal (vitamin A aldehyde). Evidence exists for low specificity of binding for RBP to various retinoid end groups (Siegenthaler, and Saurat, 1987). This finding is well supported by our recent pre-resonance Raman spectroscopic study (Manor, *et al.*, 1993), which indicates retinal is bound to RBP in much the same way as retinol (vitamin A alcohol). Moreover there was no discernible conformational change from an RBP bound retinoid in comparison to one free, in a hydrophobic environment. This result is in agreement with absorbance spectra results, displaying no change in the absorption maximum of 325 nm of retinol that is RBP bound or solvated in CCl₄. Studies of the thermodynamic parameters contributing to retinol-RBP binding (Noy, and Xu, 1990) indicate that this binding is stabilized by the entropy with little or no effect arising from binding enthalpy. It is also important to note that the X-ray studies of Cowan *et al.* (Cowan, *et al.*, 1990) indicate that the end group of the retinoid is located at the mouth of the binding pocket.

CRBP-I offers an interesting contrast to RBP in terms of its binding affinities. CRBP-I shows a clear preference for the binding of retinol over retinal (Li, *et al.*, 1990; Cheng, *et al.*, 1991). Cowan and co-workers (Cowan, *et al.*, 1990) observe the chromophore to be of a more planar conformation within the binding site of CRBP-I than in

solution. This observation is in agreement with the 25 nm red shifted absorption maximum due to retinol bound to CRBP-I. The same study indicates interactions between Lys40 of the CRBP-I and the isoprene tail of the retinol. Finally, the end group of the retinoid ligand is buried deep within the binding site.

Given these differences, a comparative vibrational spectroscopic study of binding effects upon all-*trans* retinal bound to these proteins would prove informative. Pre-resonance Raman spectroscopy of retinal bound to RBP (Manor, *et al.*, 1993) indicates that the carbonyl of the RBP bound retinal is exposed to a hydrogen bonding environment. This is indicated by a 15 wavenumber red shift and broadening of the C=O band. Several workers have demonstrated that this type of effect, first noted by Badger and Bauer (Badger, and Bauer, 1937), may be used to quantitate the strength of hydrogen bonding as a function of the carbonyl shift (Latajka, and Scheiner, 1990; Thijs, and Zeegers-Huyskens, 1984a and 1984b). The utility of this type of experiment to characterize enzyme function has been demonstrated by Tonge *et al.* and Deng *et al.* (Tonge, and Carey, 1992; Deng, *et al.*, 1994) who studied hydrogen bonding forces involved in the complexation of enzyme and substrate.

The thrust of the work presented in this study is two-fold; first, to utilize pre-resonance difference Raman spectroscopy in order to ascertain conformational effects upon all-*trans* retinal bound to CRBP-I in comparison to those of all-*trans* retinal bound to RBP. Secondly, this study uses FTIR spectroscopy to correlate the hydrogen bonding enthalpy ($-\Delta H$) to the decrease in $\nu_{C=O}$ for retinal

with a series of phenol derivatives of incremental proton donating ability. This correlation is then applied to pre-resonance Raman spectra of retinal in the binding site of RBP and CRBP-I in order to facilitate a quantitation of the degree of hydrogen bonding to the aldehyde of the retinoid in both protein-ligand complexes. A quantitative comparison of this effect in both systems would help to characterize the nature and extent of hydrogen binding forces in the binding sites of these retinal binding proteins and to the aldehyde of the bound retinal.

III. Materials and Methods

All-*trans* retinal, carbon tetrachloride, ethanol, phenol, 3,4-dimethylphenol, 3,5-dichlorophenol, 4-bromophenol, and 4-nitrophenol, (Aldrich Chemical Co., Milwaukee, WI.) were of the highest commercial grade and used without further purification. CRBP-I in the pMON expression vector was the generous gift of M. Levin (Department of Medicine, Washington University School of Medicine, St. Louis), and was expressed and purified as described earlier (Levin, *et al.*, 1990).

Protein complexes:

Concentrations of apo-CRBP-I and CRBP-I were determined from their extinction coefficients (Horwitz, and Heller, 1974; Hase, *et al.*, 1976). CRBP-I-retinal complexes were formed by mixing substoichiometric amounts of retinal (from a concentrated ethanolic

stock) with purified CRBP-I at a retinal:protein molar ratio of 0.9:1. The protein was then concentrated and ethanol removed by three consecutive washings on a Centricon 10 Centrifugal concentrator to a final protein concentration of 0.4 mM in a 20 mM sodium phosphate pH 7.4. All procedures were carried out at 4 °C in dim red light.

Spectroscopy:

Raman spectra were obtained with a Triplemate spectrometer (Spex Industries, Metuchen, N.J.) equipped with a charge coupled device (CCD) detector (LN/CCD-1152UV) cooled to -100° C and interfaced with an ST-135 CCD controller. Samples were excited by the 752 nm line from an Innova 400 krypton laser (Coherent, Palo Alto, Ca.) at 60 mW. Using the 752 nm wavelength for excitation ensured no photoisomerization of the retinal as judged from the fingerprint region of the chromophore. Residual contribution from classical (non-resonance) scattering from the protein (e.g. Amide I band and aromatic residues) was digitally subtracted as described earlier (Callender, and Deng, 1994). Spectra were acquired and analyzed on a Mac-II computer equipped using the Igor software package (Wavemetrics, OR). Spectral lines were calibrated to established Raman lines of toluene spectra. The band assignments are accurate to within ± 2 cm⁻¹ and spectral resolution was 6 cm⁻¹.

FT-IR spectra were acquired on a Digilab FTS-40 spectrometer equipped with a DTGS detector. Spectra were collected at 4 cm⁻¹ under the purge of clean and desiccated air, by signal averaging 64 - 256 scans. The spectra were apodized with a triangular function and Fourier-transformed with one level of zero filling to yield data

encoded every 2 cm^{-1} . Samples were contained in a thermostated transmission cell (Harrick Scientific, Ossining, N.Y.) equipped with KBr windows and a 0.5 mm teflon spacer. The temperature was controlled with a circulating water bath and monitored to within $\pm 0.1^\circ\text{C}$ by a digital thermocouple (Physitemp Inc, Clifton, N.J.). Three to five independent van't Hoff plots were constructed for each donor - retinal complex studied. Spectra were analyzed using software generously supplied by D. Moffat (National Research Council, Canada). Residual water vapor contribution was corrected for by subtracting water vapor spectra recorded under identical conditions. Quantitation of free OH for the phenol derivatives was accomplished by integrating the spectral area between 3540 cm^{-1} and 3635 cm^{-1} for a donor of known concentration in order to establish molar absorptivities for the molecules in question. In order to assign band positions for free and hydrogen bound retinal carbonyls in spectra where bands from hydrogen bonded retinal carbonyls significantly overlap the C=O absorption of free retinal, a spectrum of free retinal recorded under identical conditions of aperture and resolution was subtracted from that of the donor - acceptor complex.

Hydrogen Bonding Studies:

Measurements of hydrogen bonding enthalpies (ΔH) between all-*trans* retinal as acceptor and a series of phenol derivatives (phenol, 3,5-dichlorophenol, 3,4 dimethylphenol, 4-bromophenol, and 4-nitrophenol) as proton donors were performed using CCl_4 as solvent. Concentration relationships were obtained using FT-IR absorption spectroscopy to establish a ratio between the integrated areas of the

OH stretching vibrations in the region of 3600 cm^{-1} for the retinal-donor complexes to those of free donor of known concentration by the above mentioned IR spectroscopic measurements. In order to establish their equilibrium constants (K_{eq}) the following equation was applied:

$$K_{eq} = \frac{a - x}{x(b - a + x)} \quad (5.1)$$

where x is the concentration in moles/liter of free phenol as determined above, a and b are formal concentrations in moles/liter of donor and receptor respectively. These measurements were repeated at five different temperatures between $275\text{ }^{\circ}\text{K}$ and $320\text{ }^{\circ}\text{K}$. Concentrations of retinal were varied from $0 - 50\text{ mM}$, while donor concentrations were varied only in the single mM range, to prevent self association. Measurements of ΔH were then obtained plotting K_{eq} vs. $1/T$ to construct van't Hoff plots from which both ΔH and ΔS were extracted.

IV. Results

The pre-resonance Raman spectra of all-*trans* retinal in dilute CCl_4 solution and when bound to CRBP-I are shown in Figures 1a and 1b respectively. The experimental condition (protein bound chromophore $\lambda_{max} = 405\text{ nm}$; $\lambda_{laser} = 752.4\text{ nm}$) were chosen such as to minimize photo-alterations in the chromophore, while still retaining significant selective enhancement of its vibrational modes due to resonance effect. Residual scattering contribution from the

protein moiety (e.g. in the aromatic and Amide I regions) are removed from the spectra utilizing a subtraction procedure described earlier (Manor, *et al.*, 1993). Rimai *et al.* (Rimai, *et al.*, 1970a, 1970b; Gill, *et al.*, 1971; Heyde, *et al.*, 1971; Rimai, *et al.*, 1973) have shown the sensitivity of the Raman spectrum of vitamin A derivatives to the isomeric conformation of its polyene chain and the disposition of the terminal functionality. A detailed vibrational analysis of the isomers of vitamin A aldehyde was performed using normal coordinate analysis as well as site specific isotope labeling to establish assignment of the observed the Raman bands (Curry, *et al.*, 1985). These bands have traditionally been grouped into several regions according to the nature of their normal modes, and we follow this convention. In Table I we list the Raman bands which change in frequency or intensity upon binding to CRBP-I, relative to their solution spectra.

The Hydrogen Out-of-Plane Region.

Vibrational bands in the 800 - 1000 cm^{-1} region of the retinal Raman spectrum are grouped into the so called HOOP (hydrogen out of plane) region. These bands arise from out-of-plane bending vibrations of the trans-ethylenic hydrogens in the retinal polyene chain (Curry, *et al.*, 1982). Both the number of Raman bands observed in this region, and their intensity, are a sensitive measure of the degree of planarity within the chromophore. Specifically, the intensities observed at 961 and 967 cm^{-1} mostly originate from HOOP motions localized trans to the C7=C8 and C10=C11 double bonds respectively. As is seen in Figure 5.1, binding of all-*trans* retinal to

CRBP-I is accompanied by decreased intensity and resolution of these two vibrational modes. Both of these are indicative of increased planarity of the chromophore, i.e. a lower torsional angle between the polyene chain and the β -ionone ring moieties of the molecule (Eyring, *et al.*, 1982). Similar conclusions were reached from earlier absorption (Ong, and Chytil, 1978) and crystallographic measurements (Cowan, *et al.*, 1993). Importantly, the same decreased intensity is observed upon the binding of retinol (vitamin A alcohol) to CRBP-I, suggesting that both ligands are anchored in the binding pocket with similar torsional angles.

The Fingerprint Region.

The second vibrational region of interest in the spectra shown in Figure 5.1 is in the 1000 cm^{-1} and 1450 cm^{-1} frequency range, and is known as the fingerprint region. Vibrations in this frequency region originate primarily from polyene C-C stretching motions and C-H in-plane bending motions, and therefore serve as a sensitive spectroscopic marker for the isomeric conformation of the polyene chain. For example, the spectroscopic signatures of all-*trans*, 9-*cis*, 11-*cis* and 13-*cis* conformations were shown to differ drastically in this region and later used as a diagnostic tool for elucidation of the isomeric content of various retinal-based visual pigments (Oseroff, and Callender, 1974; Aton, *et al.*, 1977; Doukas, *et al.*, 1978; Pande, and Callender, 1984; Deng, and Callender, 1987). Comparison of our spectra (e.g. Figure 5.1) with those obtained using the rapid-flow techniques (Callender, *et al.*, 1976) indicate that our experimental conditions do not induce any photo-isomerization of the

chromophore, even when studying the most photo-labile isomer, 11-*cis* retinal. Comparison of the fingerprint region in Figures 5.1a and 5.1b shows that binding to CRBP-I is accompanied by changes in several fingerprint bands (see Table I), suggesting electronic and possible geometric perturbations of the polyene chain upon binding. This situation is different from the binding of all-*trans* retinal to RBP, where no changes in the fingerprint region are observed (Manor, *et al.*, 1993).

The interpretation of these changes often applies specific isotope labeling at different positions of the chromophore. From these studies, the 1163 cm^{-1} band of all-*trans* retinal found in Figure 5.1a has been assigned to C11-C12 stretch, the 1197 cm^{-1} band to C8-C9 stretch with some C9-CH₃ stretch, the 1334 cm^{-1} band to 14H rocking and the 1282 cm^{-1} band to 7H-8H rocking (Curry, *et al.*, 1985). We have drawn some conclusions and speculations from these spectra based on the previous labeling studies and the available three dimensional structure of the protein (Cowan, *et al.*, 1990; Xu, *et al.*, 1993). A few polar amino acid residues were shown by X-ray diffraction studies to reside near ($<3.6 \text{ \AA}$) the bound ligand, as well as two bound water molecules. Of particular relevance to the observed spectral changes in the fingerprint region are the proximities of Lys40 and a bound water molecule which are within $3.6 \text{ \AA}$ from the polyene chain (Xu, *et al.*, 1993). Thus, the shift of the 1163 cm^{-1} Raman band to higher frequencies can be attributed to interaction of the positively charged Lys40 with C10-C11 region of the polyene chain, while the splitting of the 1197 cm^{-1} band can be attributed to decoupling of the C9-CH₃ stretch from the C8-C9 one, possibly due to

the proximity of Q38 as well as the bound water molecule (H_2O 43). Close approach of Lysine40 and Q38 may also be responsible for the perturbation and blue shift of 14H and 7H-8H rocking modes respectively, located at 1334 cm^{-1} and 1282 cm^{-1} in the model spectrum.

The Ethylenic Region

i. C=C Vibrations

The most intense band in the Raman spectrum of virtually all retinoids is the C=C stretching mode found at 1577 cm^{-1} for all-*trans* retinal in CCl_4 (Figure 5.1a). Upon binding to CRBP-I, this band is shifted to 1565 cm^{-1} (Figure 1b, Table I). The intensity of this band arises mainly from double-bond stretching (i.e. C7=C8; C9=C10; C11=C12; C13=C14) combined with contributions from the various CCH in-plane rocking vibrations (Curry, et al., 1982). The strong resonance enhancement of this intensity stems from the delocalized nature of the conjugated double-bond system across the polyene chain. This delocalization is also the origin of the strong π - π^* transitions within the system which can be visualized in the strong electronic absorption of the molecule. Indeed, a linear correlation was shown to exist between the frequency of the ethylenic stretching vibration ($\nu_{\text{C}=\text{C}}$) and the maximal wavelength of absorption (λ_{max}) in essentially all retinoids examined (Kakitani, et al., 1983). The observed shift of the absorption maximum to higher wavelength (405 nm) and of the ethylenic stretching mode to lower frequency follows this trend qualitatively and quantitatively. The shift is best explained by the interaction of the polyene chain π system with the

positively charge of Lys40 and the polar water molecule H₂O-43. It can be noted that this interaction does not decouple any of the double bonds from the conjugated system. Interestingly, this is true only to binding of CRBP-I to retinal; when the protein is complexed with retinol, the absorption spectrum (as well as the fluorescence excitation spectrum) of the complex is split into three distinguished bands (MacDonald, and Ong, 1987; Cheng, *et al.*, 1991). The pre-resonance Raman spectrum of retinol-bound CRBP-I shows a main ethylenic vibration at 1582 cm⁻¹ with a defined, weaker band at 1625 cm⁻¹. This could be attributed to the different degrees of conjugation between the two chromophores. In retinal, the carbonyl C=O is joined to the π system of the polyene chain. In retinol, where the polar head group is C-OH, the extent of conjugation is lower, and it is therefore easier to decouple individual C=C bonds. Thus, the observed splitting in the electronic and vibrational spectra possibly reflects the extension of the conjugated system into the β -ionone ring to include the C5=C6 and C7=C8 double bonds (calculated to scatter at 1611 and 1600 cm⁻¹, respectively (Curry, *et al.*, 1985)) into the resonating π system, however their stretching vibration is decoupled from the rest of the π system which is shifted by the interaction with Lys40.

ii. The C=O Vibration

In general, C=O stretching vibrations are in the 1600 - 1900 cm⁻¹ region of the vibrational spectrum. Conjugated aldehydes, which are capable of some degree of keto-enol equilibria due to intramolecular hydrogen bonding, tend to possess less double-bond

character, and therefore be in a lower frequency region, typically 1600-1700 cm^{-1} (Colthup, *et al.*, 1990). In the absence of proteic molecules (i.e. in a dilute CCl_4 solution), the C=O stretching frequency of retinal is seen at 1664 cm^{-1} (Figure 5.1a). Interactions with proton donors further polarize the C=O bond and decrease its bond order, hence lowering its vibrational frequency. Figure 5.2 shows the pre-resonance Raman spectrum of all-*trans* retinal in the C=O stretching region (1620-1700 cm^{-1}) when dissolved in CCl_4 (spectrum A), and when bound to the two retinal binding proteins RBP and CRBP-I (spectra B and C respectively). Comparison of the protein-bound to the "free" retinal spectra reveal two major differences: first, the maximum scattering intensity is shifted to lower frequencies (1664 cm^{-1} in CCl_4 ; 1650 and 1645 cm^{-1} when bound to RBP and CRBP-I respectively). Secondly, the C=O stretching vibration broadens considerably upon binding with the proteins (FWHM= 12 cm^{-1} in CCl_4 , 24 cm^{-1} and 31 cm^{-1} when bound to RBP and CRBP-I respectively, without correction of the 6 cm^{-1} spectrometer bandpass). Both of these effects were shown in model ketones to be an outcome of hydrogen bonding of the carbonyl to the proton donors (Thijs, and Zeegers-Huyskens, 1984). In order to ascertain and quantitate this interaction, we undertook a systematic study of retinal hydrogen bonding interaction with a variety of known hydrogen bonding donors. Since the interaction involves a significant change in the dipole moment associated with the carbonyl, for accurate quantitative measurements we have utilized infra-red absorption spectroscopy, which allows accurate concentration determination using known extinction coefficients.

The interaction can be described and quantitated as the following equilibrium system:

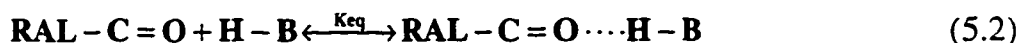


Figure 3a displays the infrared absorption spectrum of the C=O stretching region of all-*trans* retinal in CCl₄ in the presence of increasing concentrations of the hydrogen bonding donor 4-nitrophenol. As previously seen in Raman (Thijs, and Zeegers-Huyskens, 1984a) and IR (Thijs, and Zeegers-Huyskens, 1984b) studies of other model compounds, increasing proton donor concentrations results in notable red-shift of the main absorption band concomitant with significant broadening. Using known extinction coefficients for the free phenol O-H stretching region (~3650 cm⁻¹, see Materials and Methods), it is possible to quantitate the actual concentration of each species, thus allowing the treatment of the interaction as an equilibrium titration, as shown in figure 3b. It is assumed that this transition involves only two species: a free, non bonded carbonyl absorbing at 1664 cm⁻¹, and a hydrogen bonded carbonyl absorbing at 1645 cm⁻¹. This assumption is strengthened by the fact that the titration is fitted by a single binding constant (solid line in figure 5.3b) and by the observation of a single isosbestic point for the spectral transition. Thus under the experimental conditions utilized, there seems to be no contribution from side reactions which would complicate the analysis, such as self-association (Migchels, and Zeegers-Huyskens, 1991). The apparent association constant extracted from this fit is shown in Table II for complexes between all-*trans* retinal and donors of

varying acidity (phenol, 4-nitrophenol, 3,5-dichlorophenol, 3,4-dimethylphenol, and 4-bromophenol). Also listed in Table II are the spectral shifts observed upon interaction with the different donors and the reaction enthalpy as measured from the temperature dependence of the dissociation constant (van't Hoff's equation, see Materials and Methods). Further support for the notion that the observed effect is solely a result of hydrogen bonding is seen in Figure 5.4, from the linear dependence of the magnitude of the induced shift in the C=O stretching frequency and the acidity (pKa) of the hydrogen bonding donor. When the enthalpy for hydrogen bond formation is plotted *versus* the magnitude of induced frequency shift ($\Delta\nu_{\text{C=O}}$), the linear correlation shown in Figure 5.5 is obtained. The slope of this correlation (1.13 KJ/cm^{-1}) can be used as a calibration model, from which we can obtain the hydrogen bonding enthalpy given the magnitude of measured spectral shift.

Using this calibration, we find the interaction enthalpies of the carbonyl hydrogen bonding for all-*trans* retinal bound to RBP and CRBP-I (as shown in figures 5.2B and 5.2C) to be -23.5 and -28.1 KJ/mol respectively. By a similar calibration of $\Delta\nu_{\text{C=O}}$, the free energy of association (ΔG_{298}) were obtained for the hydrogen bonding of retinal's carbonyl to RBP and CRBP-I was found to be -7.2 KJ/mol and -10.5 KJ/mol. Comparing this value to the overall ΔG (as derived from published association constants) of association between all-*trans* retinal and RBP (-36.7 KJ/mol) and CRBP-I (-41.6 KJ/mol) yields the fraction of binding energy that is associated with the interaction between retinal's polar group and the binding pocket.

V. Discussion

It has been established that retinoids constitute a crucial co-factor in a variety of key physiological processes (Roberts, and Sporn, 1984). Aside from their established role as the prosthetic group of all visual photo-receptors, vitamin A derived molecules (retinal, retinol and retinoic acid) appear to be involved in many crucial biological reactions, including control of cell proliferation and differentiation (Wathne, *et al.*, 1988), and are used as therapeutic agents in many malignant conditions. All retinoids are poorly soluble in aqueous media, a fact which raised the search for their putative receptors and binding proteins. To date, two families of binding proteins were isolated from a variety of tissues which binds retinol and/or retinal with high affinity. Cellular Retinol Binding Proteins (CRBP-I and II) belong to a family of highly homologous proteins including P2 myellin protein (Jones, *et al.*, 1988), adipocyte lipid binding protein (ALBP) (Bernlohr, *et al.*, 1984), cellular retinoic acid binding proteins (CRABP I and II) (Sundelin, *et al.*, 1985; Beiley, *et al.*, 1988; Kitamoto, *et al.*, 1988) and fatty acids binding protein (FABP) (Heucheroth, *et al.*, 1987). The second family, including serum retinol binding protein (RBP) (Cowan, *et al.*, 1990; Newcomer, *et al.*, 1984), β -lactoglobulin (Sawyer, *et al.*, 1985; Monaco, *et al.*, 1987), and billin binding proteins (Holden, *et al.*, 1987; Huber, *et al.*, 1987) exhibit lower sequence homology but similar folding topology i.e. a binding cavity for the ligand composed of anti-parallel beta sheets which form a highly hydrophobic pocket for solvating the hydrocarbon moiety of the ligand. One major difference between the two protein families involves the specificity of ligand binding. While

RBP binds with similar affinity retinal, retinol and many other retinoid derivatives and isomers, CRBP-I is much more sensitive to minor changes in the ligand structure. Thus, CRBP-I binds only all-*trans* retinal and retinol and a few of their geometric isomers.

We have previously reported a vibrational study on the interactions between all-*trans* retinal and RBP, where we demonstrated that the ligand interacts with the binding pocket solely through hydrophobic interactions (Manor, *et al.*, 1993). The ligand binding pocket in RBP does not contain any charged residues, and thus binding is essentially driven by an entropic contribution of water displacement within the cavity (Manor, *et al.*, 1993; Noy, and Xu, 1990). Some geometrical changes do occur in the ligand however, as seen by the increased intensity of HOOP region vibrational modes, which suggest increased torsional strain on the polyene chain. Supporting these data, crystallographic structural determinations of RBP (Cowan, *et al.*, 1990; Newcomer, *et al.*, 1984) show that the chromophore β -ionone ring is positioned deep within the protein β barrel, the isoprene tail extended along the barrel axis toward the surface of the protein, and the end group exposed to water in the bulk phase. Accordingly, the RBP-bound retinal C=O band is seen to broaden and its frequency shifted to 1649 cm^{-1} , from a position of 1664 cm^{-1} when the chromophore is dissolved in aprotic CCl_4 . This effect has been noted previously in the spectroscopic studies of Allan and Cooper (Allan, and Cooper, 1980) and Pande *et al.* (Pande, *et al.*, 1982) where incremental quantities of water was added to all-*trans* retinal in dilute acetonitrile solutions.

The nature of interaction between all-*trans* retinoid in complex with CRBP-I is one of sharp contrast with RBP, involving a number of specific interactions between protein residues and ligand. The orientation of CRBP-I bound retinol as determined by X-ray crystallographic studies (Cowan, *et al.*, 1990; Xu, *et al.*, 1993), places the retinol OH functional group deep within the CRBP-I binding pocket, hydrogen bonded to the glutamine 108 residue. Lysine 40 of the protein makes close contact with isoprene tail, while the overall conformation of the retinoid in the binding site is planar. The pre-resonance Raman data presented in this study tend to augment and expand these assessments. Comparison of the HOOP mode region of the CCl₄ solublized and CRBP-I bound retinal spectra (800-1000 cm⁻¹ of Figures 1a and 1b respectively) indicate little or no torsional strain on the isoprene chain of the chromophore upon binding. Further agreement can be reached between X-ray and spectral data in terms of the close proximity of the lys40 side chain and the isoprene chain of the chromophore. While specific labeling would be required to isolate lys40 interactions with individual carbons along the polyene chain, it can be readily seen from comparison of fingerprint regions (1000-1450 cm⁻¹) in Figures 5.1a and 5.1b and the vibrational assignments of Palings *et al.* (Palings, *et al.*, 1985), that positions 8-12 of the molecule are affected upon ligand binding, with the perturbation centered around the 9, 10, and 11 positions upon introduction of a positive charge from the lysine. Additionally, both the X-ray data and absorption spectra agree with an interpretation of Raman data on the effect of positioning the lysine 40 with the resulting effect of shifting the λ_{max} of the bound retinal absorption

spectrum to 405 nm and the red shift of the C=C stretching band of bound retinal from 1577 cm^{-1} to 1565 cm^{-1} , a correlation noted by Kakitani *et al.* (Kakitani, *et al.*, 1983). Examination of the carbonyl region of the bound and free retinal spectra (1620-1670 cm^{-1} in Figures 5.2A and 5.2B) allows us to quantitatively isolate a single localized hydrogen bond interaction between protein and ligand in order to evaluate its contribution to the overall binding interaction between retinal and CRBP-I. The bound retinal C=O band appearing broadened at 1645 cm^{-1} clearly is being affected by a strong hydrogen bond from within the CRBP-I binding pocket, identified in the X-ray studies of Cowan *et al.* (Cowan, *et al.*, 1993) as Gln 108. The broadening of carbonyl band may in part be due to an increase in the number of degrees of freedom upon the retinal local binding environment due to the protein conformational changes. Interaction with the Gln108 side chain was rationalized as the differentiating factor in the preference of the protein for retinol over retinal (K_d of 10 and 50 nM respectively)(Li, *et al.*, 1990). Gln108, while accepting a hydrogen bond from the retinol OH functional group, must rotate and donate a hydrogen bond to the retinal C=O. The question then arises as to what portion of the overall binding interaction energy between ligand and protein is represented in this localized interaction, and how this quantitatively compares to other retinal binding proteins. To answer these questions, the frequency shift of the hydrogen bonded C=O stretching vibration as seen in the pre-resonance Raman spectrum of the protein•ligand complex has been assigned values of ΔH and ΔG derived from van't Hoff plots such as shown in Figures 5.4 and 5.5, and Table II. The value of -28.1

KJ/mol (-6.7 Kcal/mol) is obtained for the hydrogen bond between retinal C=O and CRBP-I from this relationship. This value may be compared to -23.5 KJ/mol (-5.6 Kcal/mol) for the same ligand bound to RBP, demonstrating the difference in the ΔH of hydrogen bonds between the retinal C=O and a specific amino acid or the non-specific environment of bulk water. From the relationship of $\nu_{C=O}$ to ΔG_{298} , (figure 5.4b) a ΔG_{298} value of -10.5 KJ/mol (-2.5 Kcal/mol) was obtained for the putative retinal C=O hydrogen bonding to Gln108. This value may be compared to the overall ΔG of binding value of -41.6 KJ/mol (-9.9 Kcal/mol) between retinal and CRBP-I, and ΔG of binding for retinol to CRBP-I of -45.6 KJ/mol (-10.9 Kcal/mol), as obtained from the K_d of the complex. It is clear that the hydrogen bonding interaction between the chromophore C=O functionality and the specific amino acid side chain only accounts for approximately 25% of the overall binding energy of the protein ligand complex. This result is consistent with CRBP-I binding both retinal and retinol with high affinity, yet preferentially binding retinol to retinal. This type of energetic comparison would not be relevant for retinal RBP interactions due to the difference in binding motif (see above discussion).

In summary, the pre-resonance Raman spectra presented in this study display a CRBP-bound retinal chromophore that is of planar conformation within the binding pocket of the protein, and whose isoprene tail's conjugated π electrons are perturbed for carbons at the C8 through C11 positions by the close proximity of the Lys40 residue of the protein to the chromophore. The hydrogen bonded C=O moiety accounts for a substantial (25%), but not overriding

amount of the energy of interaction, explaining both the high affinity of CRBP-I for retinal and the protein's preference for binding retinol. The bulk of the binding energy, of course, would be due to both the hydrophobic and specific interactions mentioned above. Future studies should include specific isotope labeling to further elucidate interactions between the isoprene tail of the retinal and the Lys 40, as well as similar studies of other proteins within this family to ascertain if this binding motif is conserved. It is, however, significant that the differences in binding energies between RBP and CRBP-I to retinal (-36.7 KJ/mol and -41.6 KJ/mol respectively) may certainly be accounted for largely by the differences in binding affinities of the retinal carbonyl alone in the respective protein ligand interactions. The bulk of the interactions in both complexes is, of course, hydrophobic in nature.

Table I: Vibrational modes of all-*trans* retinal which are affected by binding to CRBP-I. Frequencies are taken from Figure 1, and their assignment is based on previous labeling and theoretical studies.

Assigned Vibration	VCCl ₄ (cm ⁻¹)	VCRBP-I (cm ⁻¹)
HOOP	961;967	963; 970 weak
C10-C11 stretch	1163	1170
C8-C9 stretch	1197	1189 + 1200
7H-8H rock	1282	1299
14H rock	1334	1339
C=C	1577	1565
C=O	1664	1645

Table II.

Donor	pKa	Temp(K)	K _{form}	-ν _{C=O} (cm ⁻¹)	-ΔH (kJ/mol)	-ΔG ₂₉₈ (kJ/mol)	-ΔS ₂₉₈ (kJ/mol K)
3,4-dimethylphenol	10.32	281	16.34	12.7	19.9	5.84	0.047
		288	13.50				
		298	10.33				
		308	7.85				
		318	---				
phenol	9.95	181	32.08	13.2	21.2	7.10	0.047
		288	26.48				
		298	20.18				
		308	14.42				
		318	11.35				
4-bromophenol	9.34	182	61.91	16.4	24.6	8.71	0.053
		288	47.42				
		298	35.44				
		308	26.76				
		318	19.25				
3,5-dichlorophenol	8.18	182	164.16	17.7	26.3	11.3	0.051
		288	117.08				
		298	90.52				
		308	60.84				
		318	47.99				
4-nitrophenol	7.15	182	323.05	19.0	27.1	12.8	0.049
		288	257.27				
		298	182.22				
		308	125.62				
		318	94.05				

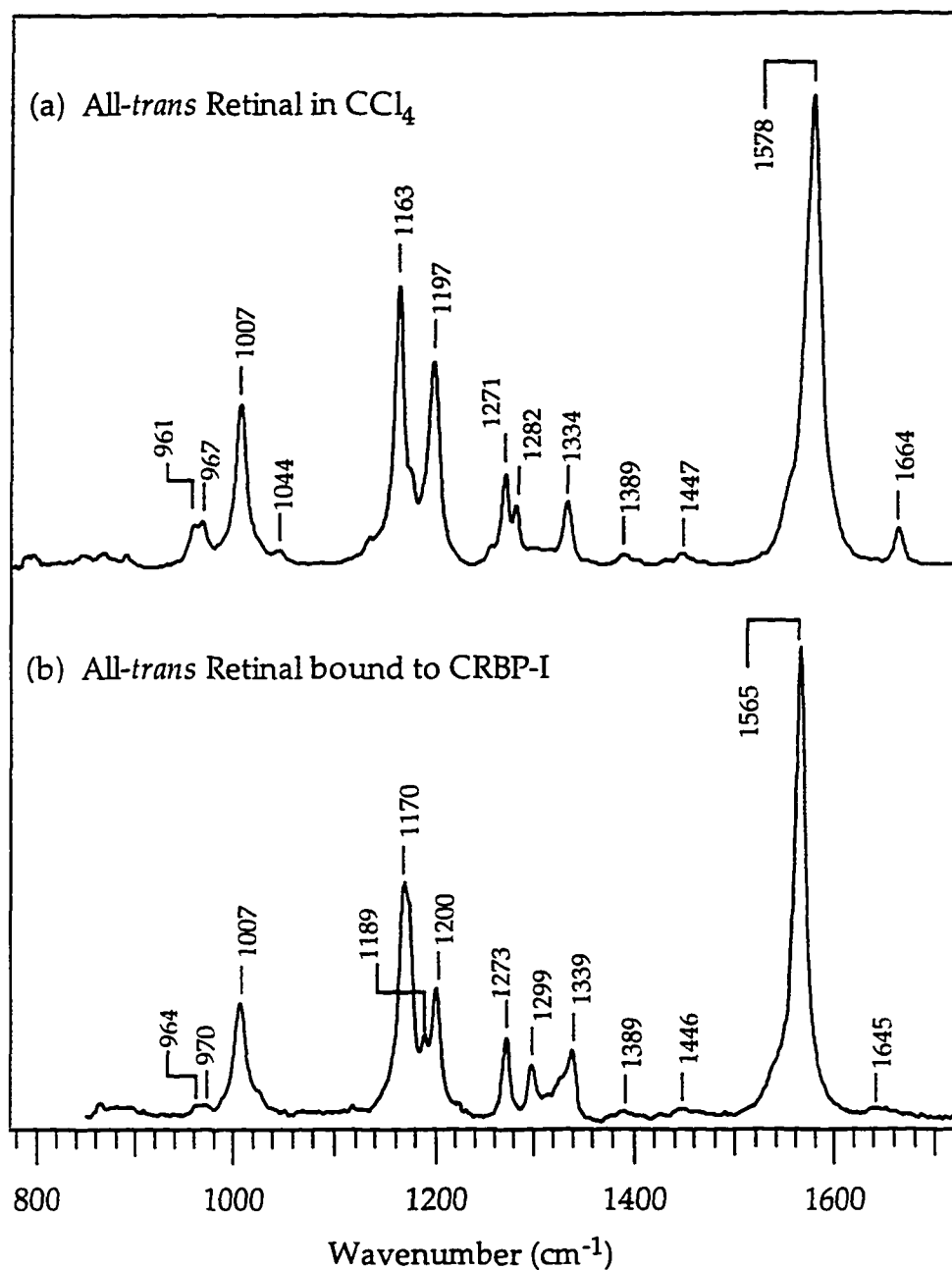


Figure 5.1 Pre-resonance Raman spectra of all-*trans* retinal in CCl_4 solution (a) and when bound to CRBP-I (b). All spectra were taken at room temperature with 60 mW excitation of 752 nm Kr+ laser line. CCl_4 and apo-protein spectra were measured under identical conditions and subtracted as described previously.

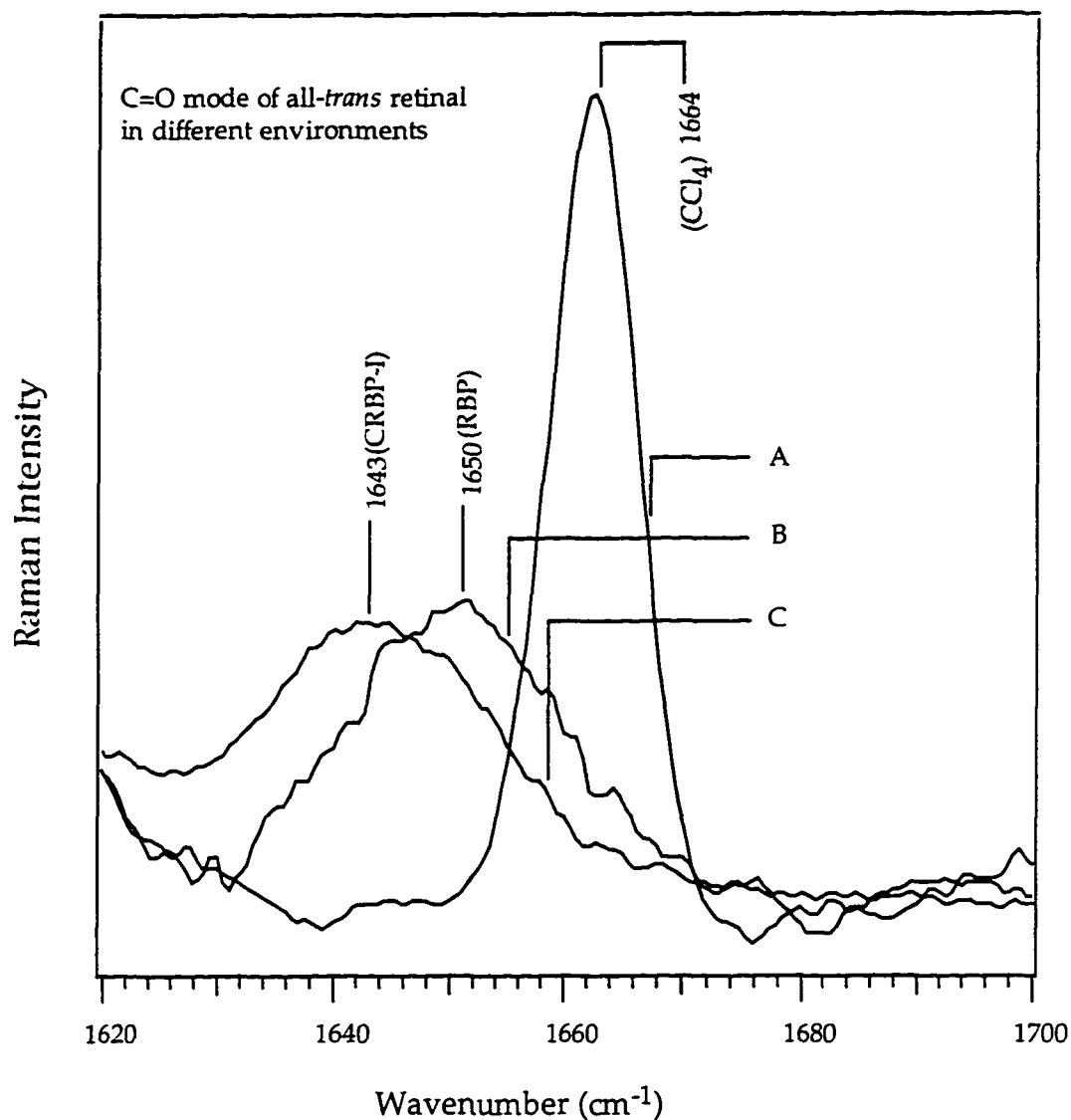


Figure 5.2 Pre-resonance Raman spectra of the carbonyl (C=O) stretching region of all-*trans* retinal in CCl₄ (0.12 mM, A) and when bound to RBP and CRBP-I (0.2 mM and 0.4 mM, B and C, respectively). Conditions are the same as in Figure 5.1

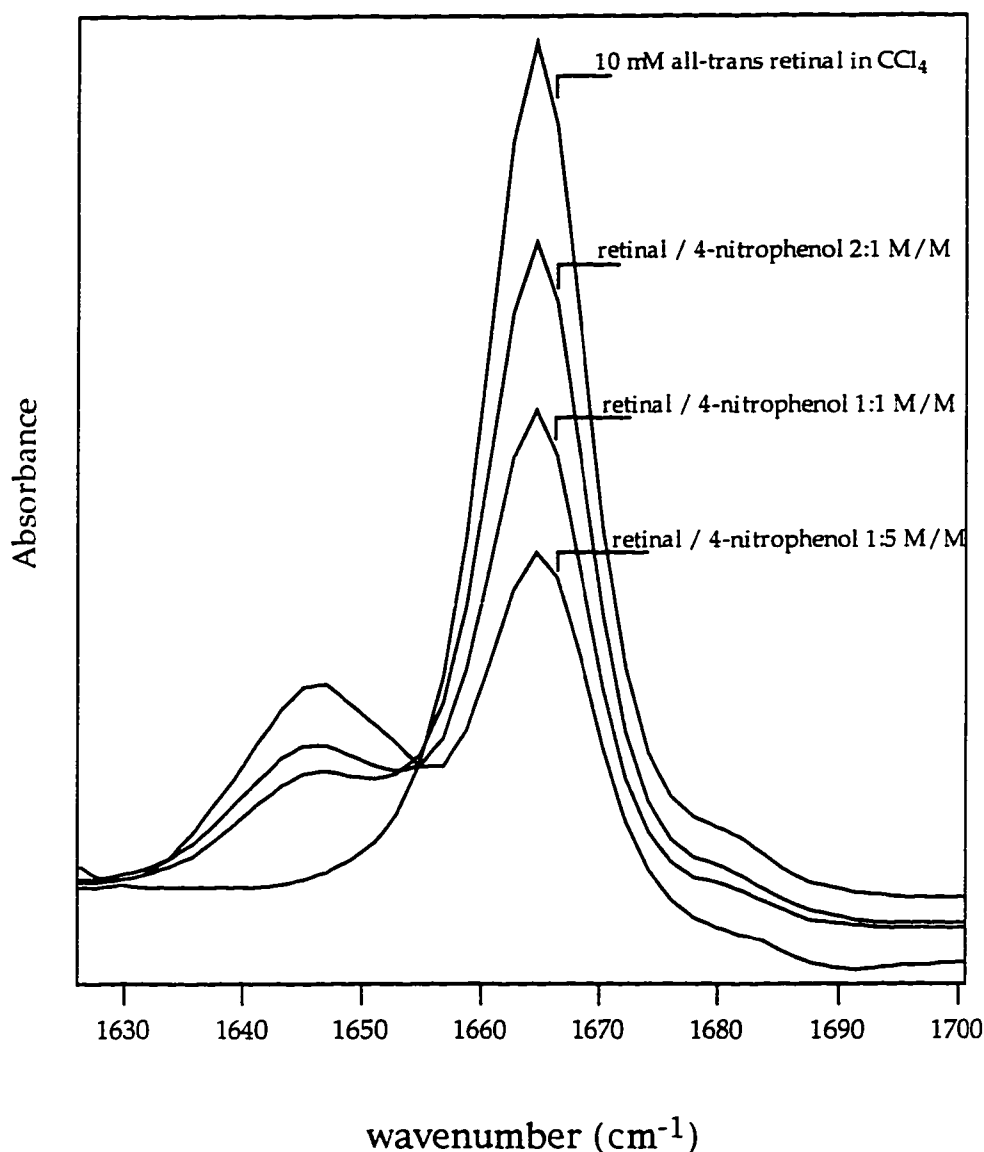


Figure 5.3a Infra-red absorption spectra of the C=O stretch region of all-*trans* retinal in the presence of increasing concentrations of the hydrogen bond donor 4-nitrophenol. Spectra of 3-7 mM all-*trans* retinal were measured as described in Material and Methods after allowing the indicated donor concentrations to equilibrate for 30 minutes at room temperature.

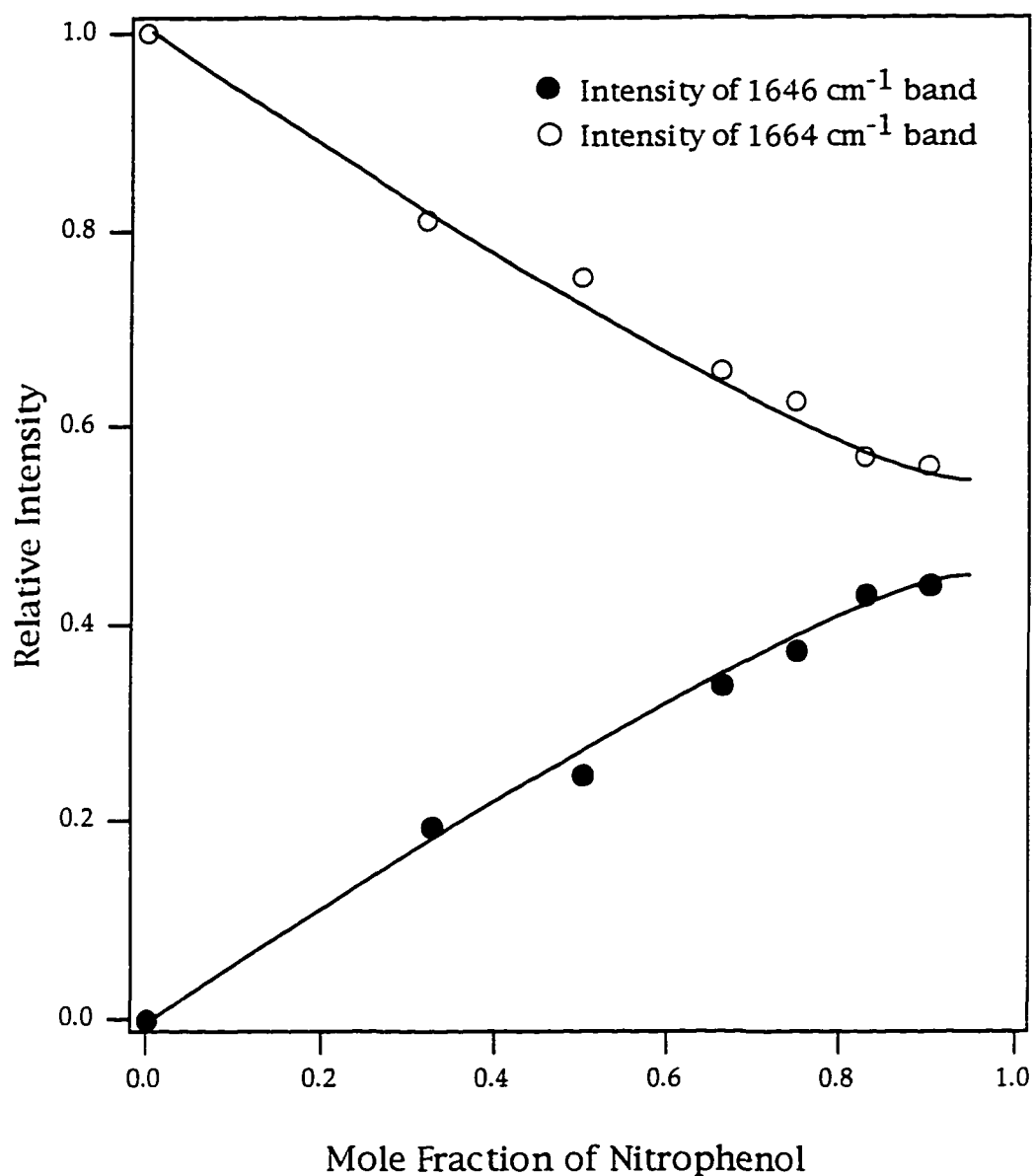


Figure 5.3b The spectral data was converted into concentrations of the H-bonded and non-bonded retinal species as described in Materials and Methods and plotted here as a function of 4-nitrophenol concentration. The solid line represents a fit of the data points to a single association constant (Eq.2).

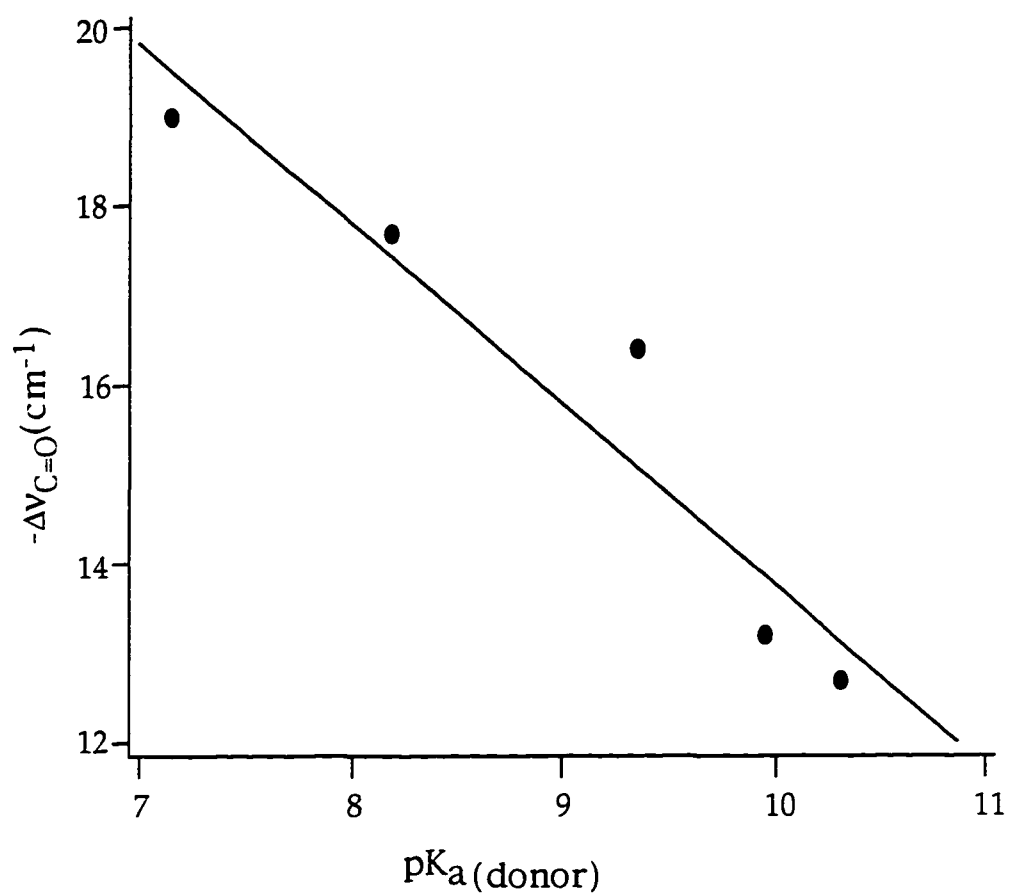


Figure 5.4 The induced shift C=O band frequency as function of hydrogen bond donor acidity (pK_a).

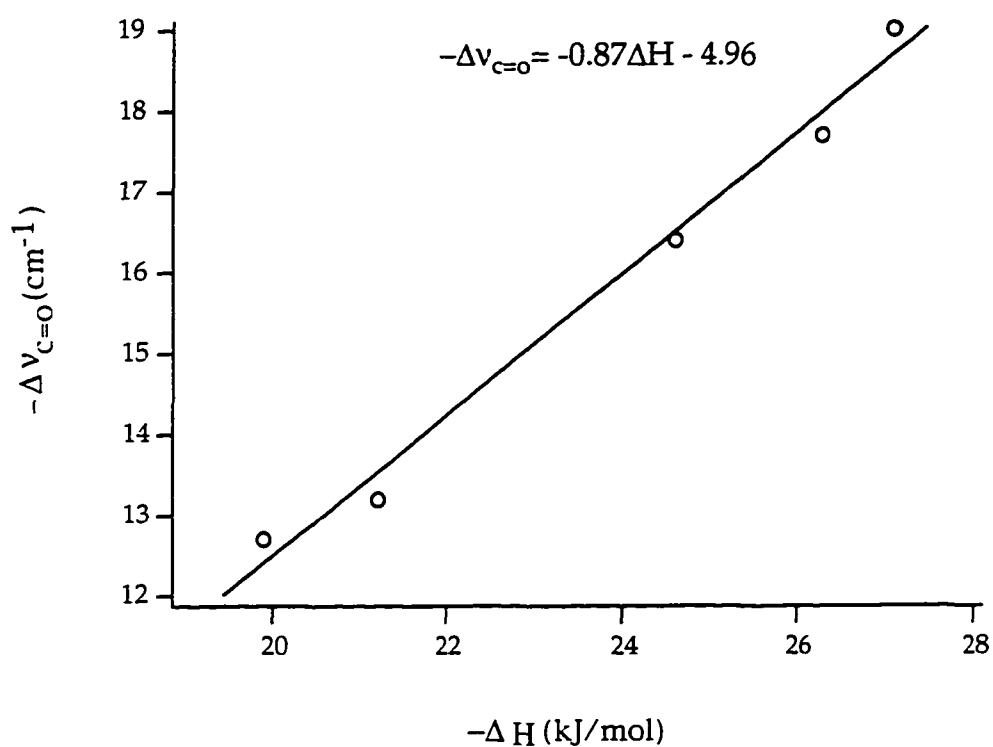


Figure 5.5a The relationship between the frequency of the carbonyl (C=O) vibrational mode and hydrogen bonding enthalpy ($-\Delta H$) of phenol derivatives, incremental in proton donating ability.

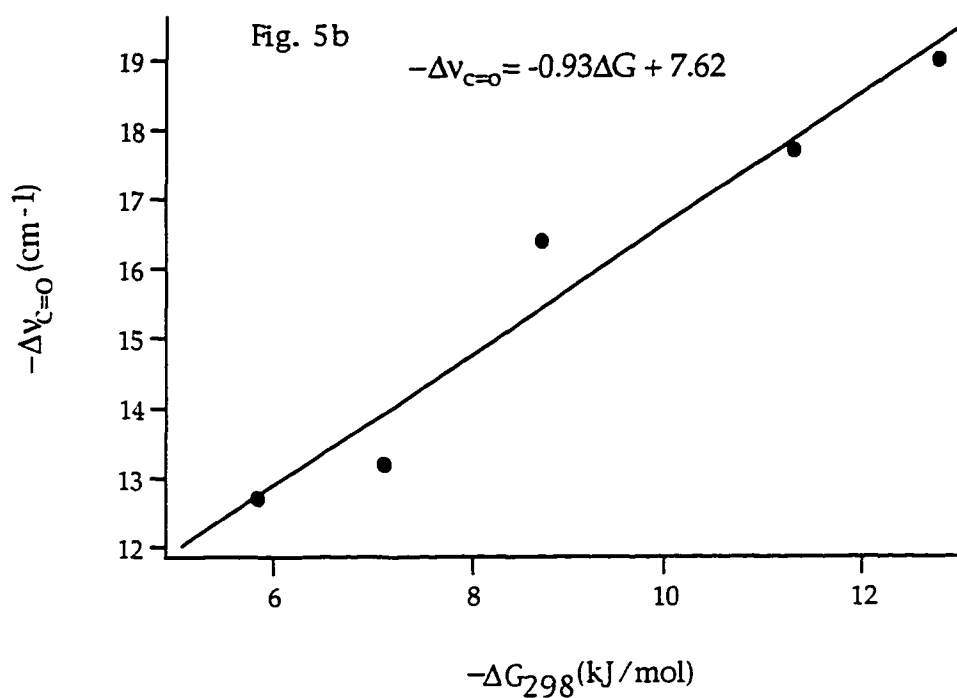


Figure 5.5b The relationship between the frequency of the carbonyl (C=O) vibrational mode and the free energy of formation at 298 K ($-\Delta G_{298}$) for the hydrogen bonding interaction between retinal and phenol derivatives of incremental proton donating ability.

Chapter 6

INVESTIGATION OF THE BINDING MECHANISM BETWEEN CRALBP AND 11-*CIS* RETINAL BY PRE-RESONANCE RAMAN SCPECTROSCOPY

I. Abstract

The pre-resonance Raman spectra of both native 11-*cis* retinal, and 11-*cis* retinal, isotopically labeled with ^{13}C at the 15 position, both complexed with cellular retinaldehyde binding protein (CRALBP) have been obtained. From these spectra a difference experiment was performed, displaying only those bands that result from the spectral shifts due to isotopic substitution. These spectra are free of contributions from the protein amide I band, that would fall within and obscure the region of the Raman spectrum ($\text{C}=\text{O}$ or $\text{C}=\text{N}$ stretch) needed to evaluate the link between 11-*cis* retinal and CRALBP. Raman spectra of 11-*cis* retinaldehyde, Schiff base, and protonated Schiff base model compounds, both native and isotopically labeled as above have been measured under the same conditions as the 11-*cis* retinal-CRALBP complexes. The same difference experiment was performed for each model compound pair. Evidence drawn from comparisons between model compound spectra and CRALBP-chromophore complex spectra, as well as an empirical

correlation between $\lambda_{\max}$ to the ethylenic (C=C) stretch, point to a possibility either Schiff base or hydrogen-bond as the linkage between CRALBP and 11-*cis* retinal. This development leads to questions regarding the enzymatic reduction of this Schiff base or hydrogen bond within the visual cycle. Our study also clearly eliminates the possibility of protonated Schiff base interaction.

II. Introduction

Cellular retinaldehyde-binding protein (CRALBP), which has only been found in light sensitive tissues (e.g. extracts of retina and retinal pigment epithelium), is unique among retinal binding proteins in its ability to specifically bind 11-*cis* and 9-*cis* isomers of retinal and retinol (Saari, and Bredberg, 1987). Upon purification from bovine retina, this protein was found in fact to contain exclusively the 11-*cis* isomers of both retinol and retinal, and no 13-*cis* or all-*trans* retinal species was detected (Futtermann, and Saary, 1977; Stubbs, *et. al.*, 1979). Such selectivity has led to speculation that this protein is an integral part of the visual cycle, serving as a stereoselective agent and a carrier substrate protein, utilized for the regeneration of 11-*cis* retinaldehyde. On the other hand, the quantum efficiency of photoisomerization for CRALBP/11-*cis* retinaldehyde ($15,400 \text{ M}^{-1} \text{ cm}^{-1}$) is only 4% that of rhodopsin, indicating a role of CRALBP in visual cycle is to protect 11-*cis* retinal from being converted to other retinoid isomers (Saary, and Bredberg, 1987).

Further evidence of the substrate nature of CRALBP-11-*cis* retinal has been shown through the specific interaction between 11-*cis*

retinol dehydrogenase of retinal pigment epithelium and this protein-aldehyde complex (Saari, and Bredberg, 1987). The CRALBP-dehydrogenase interaction is responsible for the reduction of the CRALBP bound retinaldehyde, which is otherwise inaccessible to chemical reducing agents. These results have led to speculations that conformational changes to CRALBP have been induced by the dehydrogenase, exposing the CRALBP bound aldehyde to the amino acid functional groups of the dehydrogenase enzyme without similarly exposing it to bulk solvent.

CRALBP has been cloned from both bovine and human retina, and the primary structures obtained, showing 92% identity (Crabb, *et al.*, 1988). To date, X-ray structures of CRALBP have not been obtained, nor has the nature of the link between 11-*cis* retinal and CRALBP been determined. Of the possible links between protein and chromophore, three possibilities are considered likely candidates from observation of chromophore interactions with other retinal binding proteins. They are a Schiff base (purple membrane protein of *Halobacterium halobium*: Aton, *et al.* 1977), a protonated Schiff base (Rhodopsin: Wald, 1967; Hubbard, and Kropf, 1958), and an aldehyde that is hydrogen bonded to a functional group of the protein (CRBP-I: see Chapter 5). Inaccessibility of the bound chromophore to chemical reducing agents such as NaBH_4 , NaCNBH_3 , or borane dimethylamine have made the chemical determination of this bond elusive. A possible alternative for the elucidation of the nature of this bond is vibrational spectroscopy, specifically, pre-resonance Raman spectroscopy.

Pre-resonance Raman spectroscopy is an excellent method for the examination of small molecules such as the retinal chromophore when associated with proteins. The chromophore is selectively excited by light that is coincidental with its electronic absorption. The resonantly enhanced Raman cross-section is therefore significantly larger than that of the protein, which is not similarly affected. The lower energy transition of a pre-resonance line is additionally valuable for these studies in that it does not induce photo-isomerization of the retinal chromophore. This technique may then be used as a probe of the retinal chromophore conformation and functionality, without disrupting its native physiological environment (cf. Carey, 1982; Spiro, 1987). Unfortunately, often in such studies the amide I band arising from the protein "backbone" coincides with the ethylenic region of the chromophore spectrum (crucial for the determination of the chromophore-protein linkage) in spite of the pre-resonance Raman effect. Pre-resonance Raman difference spectroscopy may then be considered as a solution to this problem.

Raman difference spectroscopy has been shown to be an effective tool many times in the study of protein-ligand interactions (see Callender, and Deng, 1994 for a review). Typically this technique involves the subtraction of the apo from the holo-protein in order to examine the ligand in its native environment, free of interferences from the protein. In the case of CRALBP, however, the apo-protein is not regenerable, leading to doubts about its conformational integrity. It is therefore not considered suitable for such spectral subtraction. In lieu of such an experiment, our work utilizes pre-resonance Raman spectroscopic studies of CRALBP bound 11-*cis* retinal that is

^{13}C labeled at the 15 position of the hydrocarbon chain, subtracted from a spectrum of native CRALBP bound retinal as a probe of the ligand to protein bond. Such a difference experiment shows only those bands obtained from the spectral shift resultant of isotopic labeling, eliminating contributions from the protein. The ^{13}C -substitution at the 15 position on the retinal hydrocarbon tail is adjacent to and therefore sensitive to the nature of the retinal functional group. This technique is then applied to model compounds of retinal aldehyde, Schiff base (SB) and protonated Schiff base (PSB) in order to ascertain the nature of the linkage between 11-*cis* retinal and CRALBP.

III. Materials and Methods

Materials:

Unlabelled 11-*cis* retinal was provided by Dr. Rosalie H. Crouch of the Medical University of South Carolina. 11-*cis* retinal, that is ^{13}C labeled at the 15 position was provided by Prof. Johan Lugtenberg of the State University of Leiden. Carbon tetrachloride, ethanol, methanol, and hydrochloric acid, purchased from Sigma Chemical Co. (St. Louis, Mo.) were of the highest commercial grade and used without further purification. Distillation of n-butylamine (Sigma) was performed prior to any SB or PSB synthesis experiment.

Methods:

CRALBP Studies:

CRALBP complexed with either unlabeled or ^{13}C labeled 11-*cis* retinal were expressed in, and isolated from *Escherichia coli* clones

according to the method of Saari, and Bredberg, (1987). CRALBP concentrations were determined from their absorption coefficients (Hase, *et al.*, 1976; Horwitz, and Heller, 1974) CRALBP was concentrated to 0.54 mM in 20mM phosphate buffer, pH 7.4 for spectroscopic measurements on a Centricon 10 centrifugal concentrator.

Raman spectra of CRALBP complexes were obtained with a Spex Triplemate spectrometer (Spex Industries, Metuchen, N.J.) equipped with a charge coupled device (CCD) detector (LN/CCD-1152UV), cooled to -100° C, and interfaced with an ST-135 CCD controller. Laser excitation energy was varied from 60 to 100 mW. Both 676 and 752 nm laser lines were provided by an Innova 400 Krypton Ion laser (Coherent Laser Group, Palo Alto, Ca.). The choice of either line would selectively excite the the bound 11-*cis* retinal without similarly affecting the protein, minimizing its contribution to the spectrum. The same lines insure no photoisomerization of the bound retinal chromophore would take place. Difference spectra between CRALBP complexed with both labeled and unlabeled 11-*cis* retinal were achieved with a special split chamber Raman cell developed specifically by our laboratory earlier for Raman difference spectroscopy. Spectra were aquired and analyzed on a Mac-II computer, equipped with Igor software (Apple, Cupertino, Ca.). Spectral lines were calibrated to established Raman lines of toluene spectra. The band assignments are accurate to within ± 2 cm⁻¹ and spectral resolution was 6 cm⁻¹. The sample volume for these experiments was ~30 μ l.

Model Compound Studies:

$^{15}\text{-}^{13}\text{C}$ labeled and unlabeled 11-*cis* retinal were treated equivalently. Schiff bases and protonated Schiff bases were prepared according to the method of Palings, *et al.* (1987). Briefly, the Schiff Base (SB) was obtained through the reaction of the 11-*cis* retinal with a 15-20 molar excess of n-butylamine in 1 ml of hexane for 20-30 minutes. Both hexane and excess n-butylamine were evaporated under a stream of nitrogen gas. The resultant SB was then dissolved in 10 ml of CCl_4 , where it was retained for spectroscopic study or converted to a protonated Schiff Base (PSB). Conversion to a PSB requires that CCl_4 be replaced by methanol as solvent, due to the instability of the 11-*cis* PSB in CCl_4 . The SB solution was titrated with aliquots of an HCl acidified methanol solution, and monitored by absorption spectroscopy for the completeness of the reaction. Aliquots of the acid-methanol solution should be reasonably small (100-200 μl) and in order to insure no thermal isomerization resulting from the heat of the reaction would occur. All procedures are carried out in dark or in dim red light. Raman spectra for the model compounds were obtained using the spectroscopic methods as stated above for CRALBP. Samples were concentrated under a stream of nitrogen gas to $\sim 1\text{mM}$ as determined by absorption spectroscopy. The sample volume for these experiments was $\sim 30\ \mu\text{l}$.

IV. Results

The position of λ_{max} in retinoids has been determined to be primarily a function of the degree of electron localization of in the

conjugated polyene chain, that would affect the bond order and length, and hence the stretching frequency of the ethylenic (C=C) band. Figure 6.1 presents an empirical relationship of the ethylenic band position and the frequency shift of $\lambda_{\max}$ for retinal related chromophores, specifically: aldehyde, Schiff base, and protonated Schiff base. The compounds used to establish this relationship have been drawn from several sources (Manor, *et al.*, 1993; Palings, *et al.*, 1987; Kakitani, *et al.*, 1983; Aton, *et al.*, 1979; Doukas, *et al.*, 1978; Aton, *et al.*, 1977). One definite point to be obtained from this exercise is that no known protonated Schiff base absorbs below a $\lambda_{\max}$ of 440 nm. The 420-425 nm maximum observed for the CRALBP bound chromophore would therefore be more consistent with that of either an aldehyde or a Schiff base rather than a protonated Schiff base.

The inability to obtain an apo-protein that retains the conformational integrity of the holo-CRALBP eliminates the option of using difference spectra that subtracts apo from holo-protein in order to examine the linkage between the CRALBP and its bound retinal ligand. Both C=O and C=N stretching vibrations fall within the range of the Amide I band arising from the protein. Without an apo-protein spectrum for subtraction, analysis of this region would prove problematic. To circumvent this difficulty, a difference experiment, subtracting the spectrum of complexed CRALBP bound to 11-*cis* retinal, that was ^{13}C labeled at the 15 position, from the spectrum of an unlabeled 11-*cis* retinal-CRALBP complex was performed. The resulting spectrum would cancel out contributions from the protein, especially the Amide-I band which undergoes negligible alteration

due to the binding of the retinal chromophore, displaying only those bands of retinal ligand affected by the labels. The results of this experiment are presented in Figure 6.2. The overlaid spectra over the range of $1400\text{--}1700\text{ cm}^{-1}$ are shown in 6.2a. and the resultant difference spectrum in figure 6.2b. Labeling at the 15 position results in a frequency shifts for the functional group ($\text{C}=\text{O}$ or $\text{C}=\text{N}$) as well as for some modes of the collective $\text{C}=\text{C}$ stretching mode that results in some uncoupling (e.g. the $\text{C14}=\text{C13}$ stretching). The functional group vibration in the unlabeled 11-*cis* CRALBP complex is a positive band falling at 1628 cm^{-1} , while a negative $15\text{-}^{13}\text{C}$ labeled band is found at 1614 cm^{-1} in the derivative spectrum. This spectrum may then be used as a benchmark by which to compare model compound difference spectra of $15\text{-}^{13}\text{C}$ labeled and unlabeled 11-*cis* retinal as well as the Schiff base and the protonated Schiff base.

Figure 6.3a, b, and c display the ethylenic region in the spectra of CCl_4 solublized native 11-*cis* retinal, $15\text{-}^{13}\text{C}$ labeled 11-*cis* retinal and their resultant difference spectrum, respectively. Carbonyl band frequencies fall at $\sim 1666\text{ cm}^{-1}$ for native 11-*cis* retinal and $\sim 1628\text{ cm}^{-1}$ for $15\text{-}^{13}\text{C}=\text{O}$ labeled retinal. Their difference spectrum yields a positive negative pair at these frequencies, as well as a pair at 1578 and 1595 cm^{-1} that is resultant of $\text{C}=\text{C}$ uncoupling due to the isotopic substitution. If an aldehyde band are present in the spectrum of the CRALBP bound retinal, one would expect a positive-negative pair similar to the 1664 and 1629 cm^{-1} bands, shifted to somewhat lower frequencies as a result of hydrogen bonding interactions (Manor, *et al.*, 1993). If this is the case, the corresponding unlabeled $\text{C}=\text{O}$

stretching mode in the CRALBP-retinal complex spectrum falls at 1628 cm^{-1} , about 38 cm^{-1} redshift from that in a non hydrogen bonding environment. The less downshift for the ^{13}C -labeled carbonyl stretch is due to the isotopic effect, which causes more conjugation between C=O and C=C modes. According to our previous hydrogen bonding studies (see section 5), this putative local ligand-protein interaction gives rise to -11.8 kcal/mol as part of the total binding energy. This is a very strong hydrogen bonding interaction, compared to the other two proteins we have investigated, RBP and CRBP-I, whose hydrogen bonding interaction enthalpies are -6.7 kcal/mol and -5.6 kcal/mol , respectively. In the spectroscopic studies employing Badger-Bauer relationships we have done so far on the interactions between retinal carbonyl group and hydrogen bond donors from different phenol derivatives, such as phenol, 3,4 dimethylphenol, 4-bromophenol, 3,5-dichlorophenol and 4--nitrophenol, we observed that the range of interaction enthalpy for these species falls between -4.8 to -6.5 kcal/mol . Therefore the value of -11.8 kcal/mol in the case of CRALBP seems too large as a normal hydrogen bond. However, it is not a surprise if the hydrogen bonding donor comes from a charged group on a polypeptide side chain. This occurs for DABA bound to LADH and Pyruvate adduct in LDH, where local interaction enthalpies as high as -14 kcal/mol were found (Callender, and Deng, 1994). Thus, it seems feasible that there exists a linkage as the free aldehyde hydrogen bonding to a charged group on a CRALBP amino acid residue at the binding site.

Figure 6.4 treats the spectra arising from the PSB of retinal in much the same way as Figure 6.3. Again Figure 6.4a, b, and c

correspond to the native, labeled and difference spectra of the retinal PSB respectively. The C=N stretching frequency lies at 1658 cm^{-1} for the unlabeled retinal and at 1638 cm^{-1} for the $15\text{-}^{13}\text{C}$ labeled chromophore. The net result of the difference spectrum is very similar to that of the free aldehyde. The 1628 cm^{-1} band in the CRALBP/11-*cis* retinal complex is again significantly lower in frequency than that of the corresponding band in the model compound spectrum, leaving the possibility a PSB linkage between the retinal and protein also an unlikely choice.

The set of model compound Schiff base spectra are shown in Figure 6.5 (6.5a, native 11-*cis* retinal; 6.5b, $15\text{-}^{13}\text{C}$ labeled 11-*cis* retinal; and 6.5c, difference spectrum). This difference spectrum comes closest to resembling that of the 11-*cis* retinal-CRALBP complex both in terms of band positions and the overall spectral contour. The 1621 cm^{-1} position of the band arising from the functional group in the model compound is a reasonable match for the corresponding band in the protein-ligand complex. Additionally, a comparison of the contours of these spectra show similarities not only in the functional group, but the C=C stretching modes as well. Thus the labeling affect on the Schiff base (SB) provides by far the best match between any of the model compounds and the 11-*cis* retinal-CRALBP complex.

Figure 6.6a and b display the "fingerprint" region of the Raman difference spectra of 11-*cis* retinal-CRALBP complex and 11-*cis* retinal Schiff base model compound, respectively. The CRALBP difference spectrum displays a positive-negative band pair at 1159 and 1152 cm^{-1} , while the model compounds display a similar pair at

1161 and 1150 cm^{-1} . Comparison of the two spectra shows a good amount of subtle detail arising from labeling shifts in the model compound spectrum is not present in the CRALBP difference spectrum. This may be in part due to conformational restraints placed upon the chromophore by the protein. The 1159-1152 cm^{-1} difference band, however persists. This band has been assigned to the C₁₄-C₁₅ stretch in retinal according to labeling studies performed by Palings, *et al* (1987). This C-C bond is adjacent to and most affected by the retinal functional group and hence, the nature of the bond between protein and chromophore.

V. Discussion

The identification of the linkage between 11-*cis* retinal and CRALBP has proven to be a difficult undertaking, providing some ambiguous results along the way. Attempts to reduce 11-*cis* retinal bound to CRALBP by chemical means have proven to be ineffective (Saari, and Bredberg, 1982), attesting to the inaccessibility of the retinoid to chemical reducing agents. The same study, however shows the bound ligand in this complex to be accessible to enzymatic reduction by an 11-*cis* retinol dehydrogenase retaining its 11-*cis* conformation. The product of this reduction remained bound to CRALBP. The above mentioned study indicates that there are conformational changes to CRALBP as a result of the specific interactions with the dehydrogenase, that allows accessibility of the bound chromophore for reduction that would not be available to typical chemical reducing agents. Do in part to this difficulty of chromophore accessibility, development of chemical methodologies to determine the nature of the linkage between the bound chromophore

and protein have remained elusive. Spectroscopic evidence, therefore provides some of the most compelling evidence in elucidating the nature of this interaction. The pre-resonance difference Raman spectroscopic data presented in this study, along with absorption spectral information provide the basis by which a process of elimination may be utilized in order to help establish the nature of the 11-*cis* retinal to CRALBP bond. This study considers three candidates as possible links. These are; an aldehyde, Schiff base, or protonated Schiff base linkage between protein and chromophore.

The link between 11-*cis* retinal and rhodopsin is well known as a protonated Schiff base (Methies, *et al.*, 1977). For this reason one of the foremost logical possibilities to be considered for 11-*cis* retinal to CRALBP complexation would be such a bond. The data in Figure 6.1 is a reasonably linear empirical correlation between the frequency shift of the ethylenic (C=C) band and that of λ_{max} for a series of retinoids that are either aldehyde, Schiff base (SB) or protonated Schiff base (PSB). Both the ethylenic frequency shift and absorption maximum of the retinoids plotted are based upon the degree of electron localization within the essential C=C backbone of the retinoid as affected by the electron withdrawing nature of the functionality involved. There is a good deal of overlap in positions of aldehydes and Schiff bases in this plot which covers the range of absorbance maxima between 350-425 nm. No known protonated Schiff base, however, absorbs below 440 nm. This information may then be coupled to information obtained through the comparison of the difference spectra in Figures 6.2b and 6.4c. The difference spectrum

in Figure 6.2b results from the subtraction of 11-*cis* retinal ^{13}C labeled at the 15 position complexed to CRALBP from that of native 11-*cis* retinal complexed with CRALBP. The key band is one arising at 1628 cm^{-1} . The difference spectrum in Figure 6.4c is resultant of model compound PSBs labeled in the same way as the protein complexes. The band arising from the protonated $\text{C}=\text{N}$ stretch falls at a characteristic frequency of 1658 cm^{-1} . The discrepancy between this signature band in both complexes combined with the λ_{max} to ethylenic frequency correlation would render a protonated Schiff base (PSB) as an extremely unlikely candidate as the link between protein and chromophore.

In some cases the retinal chromophore has been found to retain an aldehyde functionality upon complexation with a retinal binding protein. One such an instance has been reported by Manor, *et al.* (1993) for retinal complexing to RBP. This study reports a red shift of the retinal carbonyl band to 1650 cm^{-1} upon RBP complexation by comparison to 1664 cm^{-1} in CCl_4 . This shift in the vibrational frequency has been ascribed to hydrogen bonding. A proton donor in the vicinity of the retinal $\text{C}=\text{O}$ stretch will draw electrons toward the oxygen atom. This interaction polarizes the $\text{C}=\text{O}$ bond, decreasing the bond order, hence lowering the vibrational frequency. Several studies have in fact correlated a $\nu_{\text{C}=\text{O}}$ shift to hydrogen bonding enthalpy in small molecules (Latajka, and Scheiner, 1990; Thijs, and Zeegers-Huyskens, 1984). Comparison of the difference spectrum of the 15- ^{13}C labeled and native retinaldehyde model compounds in Figure 6.3c to the CRALBP-retinal difference spectrum in Figure 6.2b shows a discrepancy between these spectra in the region of the

stretching vibration of the functional group. The model compound spectra display a positive-negative pair of bands at 1666 and 1629 cm^{-1} , arising from the C=O stretching vibrations of the native and subtracted 15- ^{13}C labeled compounds, respectively. The corresponding bands in the CRALBP spectra in Figure 6.2b fall at 1628 and 1614 cm^{-1} , respectively. The value of 1628 cm^{-1} would agree with that of a C=O stretching vibration in a very strong hydrogen bonding interaction, possibly with a charged group along the protein side residue at the binding pocket. Based on our Badger-Bauer relationship study, we determined the local enthalpy due to this hydrogen bond is -11.8 kcal/mol.

Even though the process of elimination cannot exclude the possibility of a hydrogen bonding interaction between CRALBP and 11-*cis* retinaldehyde, the combination of the experimental results achieved so far points to a Schiff base (SB) as the best candidate for the link between 11-*cis* retinal and CRALBP. There are four pieces of spectroscopic data that would support such a choice: 1). 11-*cis* retinal bound to CRALBP has an absorbance maximum at ~420-425 nm in a plot of the ethylenic C=C shift against λ_{max} in Figure 6.1, supportive of either a Schiff base or an aldehyde, (No protonated Schiff base has been found with a λ_{max} below 440 nm.). 2). Comparison of the ethylenic region in the pre-resonance Raman difference spectra of the Schiff base model compounds and the CRALBP complexes, as presented in Figures 6.5c and 6.2b respectively, show remarkable similarity in their spectral contours. The 1621 cm^{-1} band in the CRALBP complex matches well with the corresponding functional group band in the SB model compounds. 3). The C=C stretching

vibrations falling from 1550 to 1610 cm^{-1} in the difference spectra also show excellent similarity in contour between model compounds and protein-ligand complex. Figure 6.6 displays all of the difference spectra in the ethylenic-Schiff base region (1400-1700 cm^{-1}) for comparison. 4). The case for a Schiff base linkage may be further augmented by comparison of the "fingerprint" region between SB model compounds and the CRALBP complexes (Figures 6.7a and 6.7b, respectively). In Figure 6.7a a pair of positive-negative bands present at 1161 and 1152 cm^{-1} appears in the difference spectrum of the CRALBP complexes. A similar pair of bands arises only in the analogous Schiff base difference spectrum in Figure 6.7b. This band has been attributed to the C₁₄-C₁₅ stretching mode as a result of the labeling studies done by Palings, *et al.* (1987). This band therefore been determined to arise from the stretching mode that is adjacent and hence most affected by the electron withdrawing nature of the functional group of the chromophore, displaying bands consistent for both the CRALBP complex and Schiff base model compound difference spectra. Also shown in Figure 6.7 are the the difference spectra of Aldehyde and PSB, but they do not resemble the similar pattern as that of CRALBP. From this reasoning the pre-resonance Raman spectroscopic evidence for a Schiff base linkage between 11-*cis* retinal and CRALBP would appear to be compelling.

Despite the spectroscopic evidence, reduction and entrapment of the putative Schiff base by chemical means has remained elusive (Unpublished data of Saary and Bredburg). Experiments performed have attempted to subject ^{14}C labeled 11-*cis* retinaldehyde complexed to CRALBP to chemical reduction. It was assumed that

such a reduction would covalently bind the retinal Schiff base to the protein, rendering the chromophore unavailable to extraction in an organic solvent. Upon treatment with a reducing agent (e.g. sodium borohydride, borane dimethylamine, pyridine/borane etc.), followed by treatment of the complex with a denaturant such as 1% SDS, 1% Triton TX-100, or 8M urea, the retinal remained extractable into an organic phase such as hexane. Additionally the possibility that the Schiff base was being reduced between the retinaldehyde and an amino acid containing component, unattached to the protein was also investigated by HPLC. This assay revealed only extracted retinol, with no secondary amine observable. Attempts to use anhydrous conditions for reduction have also produced ambiguous results at best.

The remaining problem is then to reconcile the apparent difficulty in chemically reducing and trapping the putative Schiff base with convincing evidence obtained from pre-resonance Raman spectroscopy that such a link exists between 11-*cis* retinal and CRALBP. One possible explanation suggests an environment of the Schiff base resulting in a rate of hydrolysis faster than the rate of reduction. The existence of such a bond between ligand and protein leads to questions pertaining to interactions between CRALBP and retinoid metabolizing enzymes. For example, what is the mechanism of reduction of the Schiff base bound retinaldehyde in CRALBP? If the enzyme reduces the bound substrate, why have chemical reducing agents failed? Further spectroscopic studies will prove valuable in determining the nature of some of the interactions between CRALBP and retinoid metabolizing proteins as well as

further elucidating the nature of the chromophore-protein bonds and chromophore conformation in such instances.

The next step in the investigation on the binding mechanism between CRALBP and 11-*cis* retinal will focus on the final decision out of the choices between hydrogen bonding and Schiff Base linkages. This ambiguity can be tackled by the isotopic labeling experiment with ^{18}O edited 11-*cis* retinal. The feasibility of this experiment is based on the analysis described as following,

a. If the interaction is through a Schiff base bridge, the isotopic labeling will not affect the Raman spectroscopic results as obtained before, because the labeled carbonyl oxygen atom is replaced by the nitrogen atom of a lysine residue of CRALBP which constitutes the C=N bond.

b. However, if the retinoid ligand is in its aldehyde form hydrogen bonding to CRALBP, the carbonyl C=O stretch will undergo redshifting due to mass effect on the vibrational frequency. Thus one expect a modified Raman difference spectrum in which both 1628 cm^{-1} and 1614 cm^{-1} bands are moved to a lower frequency position, indicating the ^{18}O -isotopically edited oxygen atom at the 11-*cis* retinal terminal group is involved as an integral part of C=O...X bond.

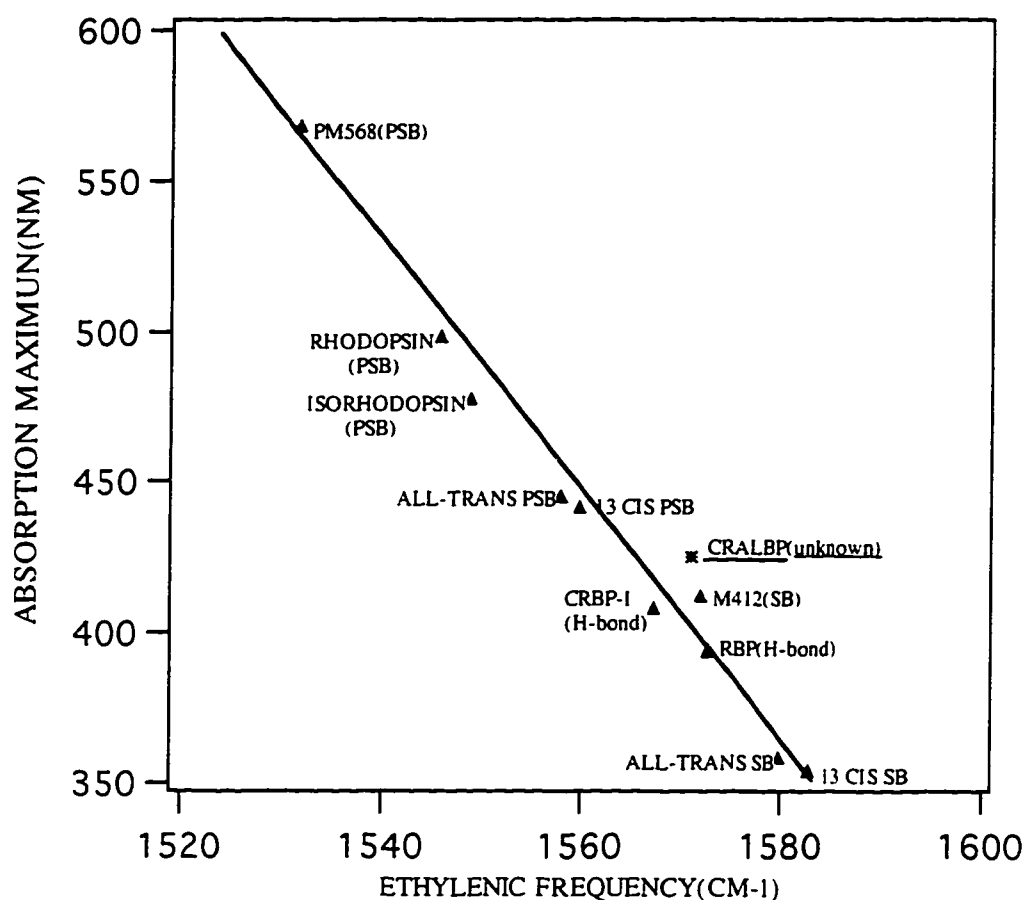


Figure 6.1 Correlation of the ethylenic (C=C) stretching frequency of a series retinal, aldehyde, Schiff base (SB), and Protonated Schiff base (PSB) to their absorption maxima ($\lambda_{\max}$). The position of retinal bound to CRALBP is marked with asterisk. Data are pooled from several sources (see results in the text).

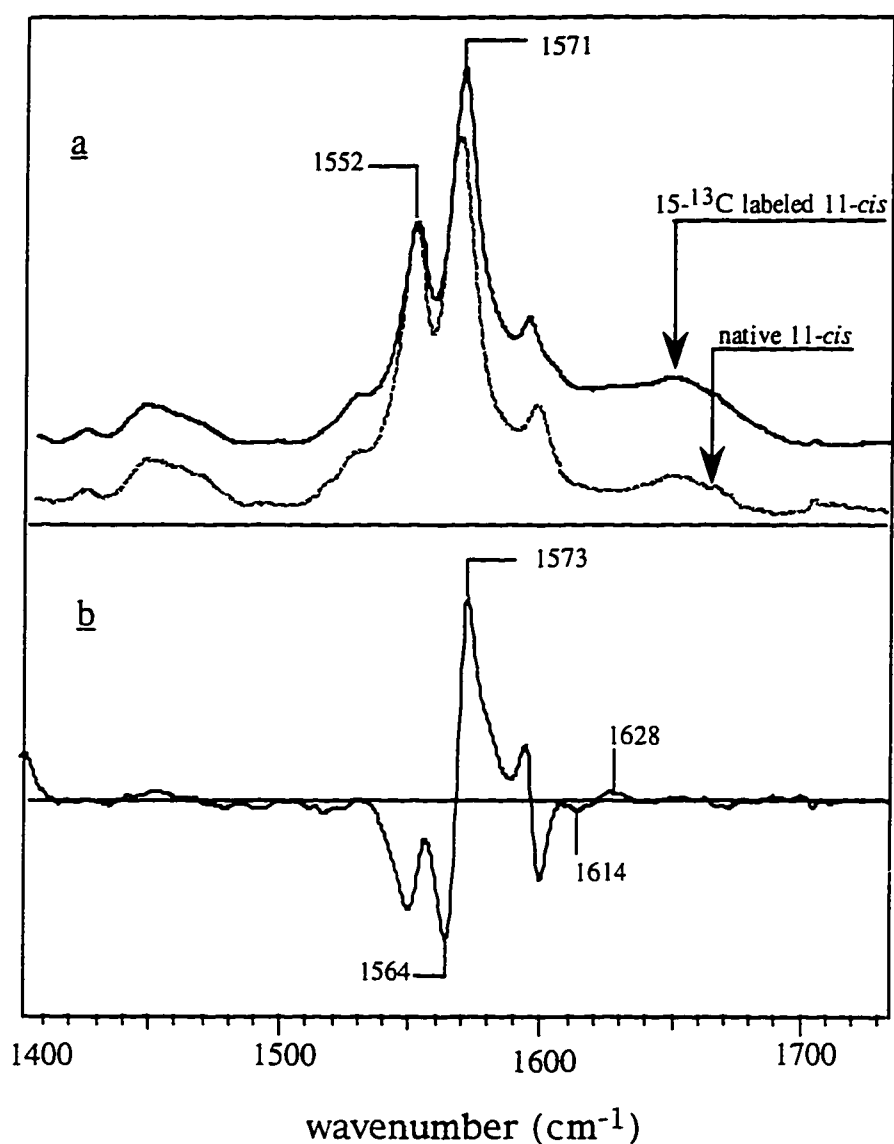


Figure 6.2 a). Pre-resonance Raman spectra of native 11-*cis* retinal complexed with CRA1BP overlaid with 11-*cis* retinal ^{13}C labeled at the 15 position complexed with CRA1BP in the range of 1500-1700 cm^{-1} . b). Difference spectrum resulting from the subtraction of the 15- ^{13}C labeled 11-*cis* retinal complexed with CRA1BP spectrum subtracted from a spectrum of native 11-*cis* retinal complexed with CRA1BP.

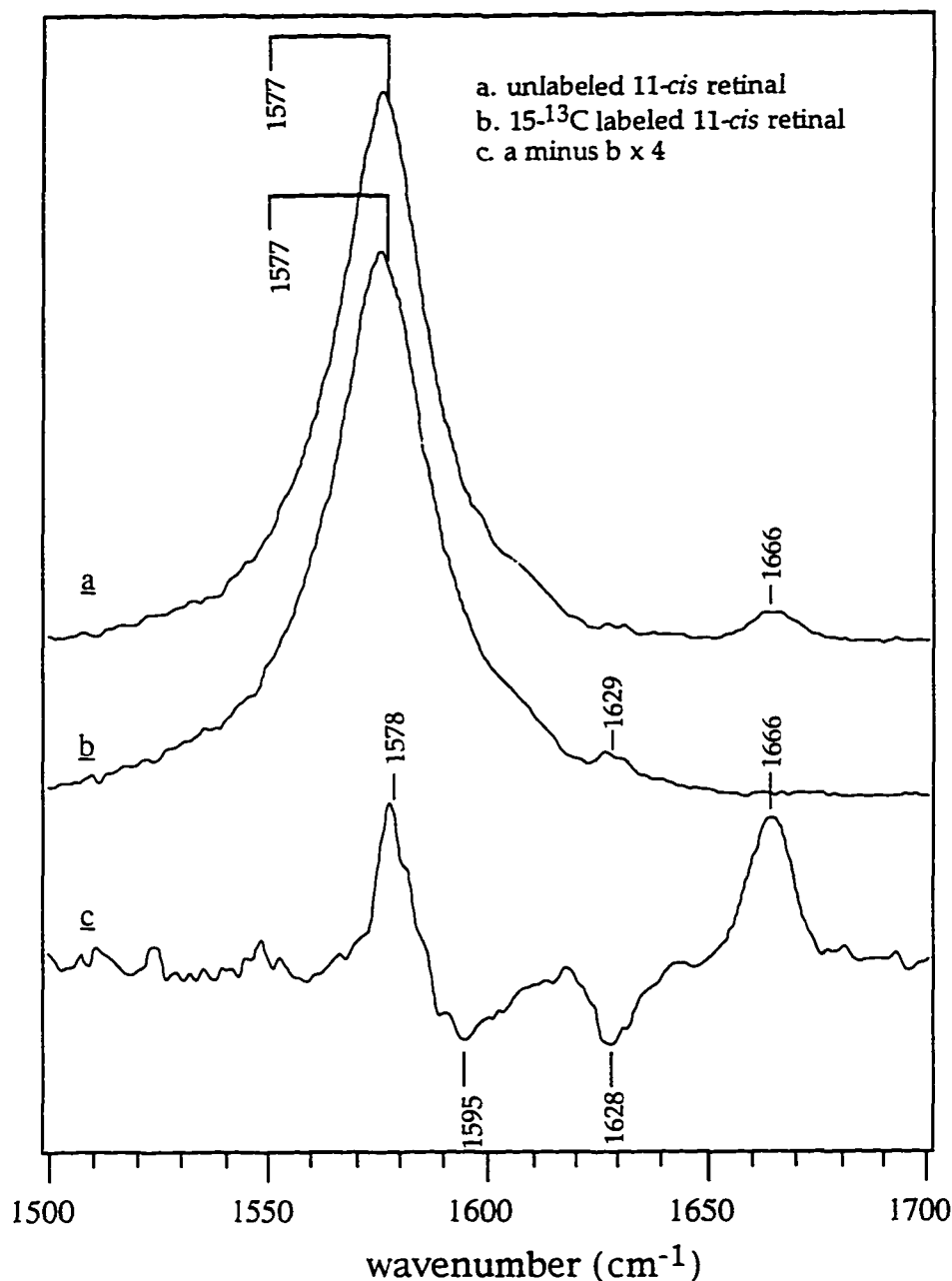


Figure 6.3 a). Pre-resonance Raman spectrum of 11-*cis* retinal solubilized in CCl_4 in the region of 1500-1700 cm^{-1} . 3b). pre-resonance Raman spectrum of $^{15}\text{-}^{13}\text{C}$ labeled 11-*cis* retinal solubilized in CCl_4 in the region of 1500-1700 cm^{-1} . 3c). Difference spectrum resulting from the subtraction of the spectrum of b from that of a.

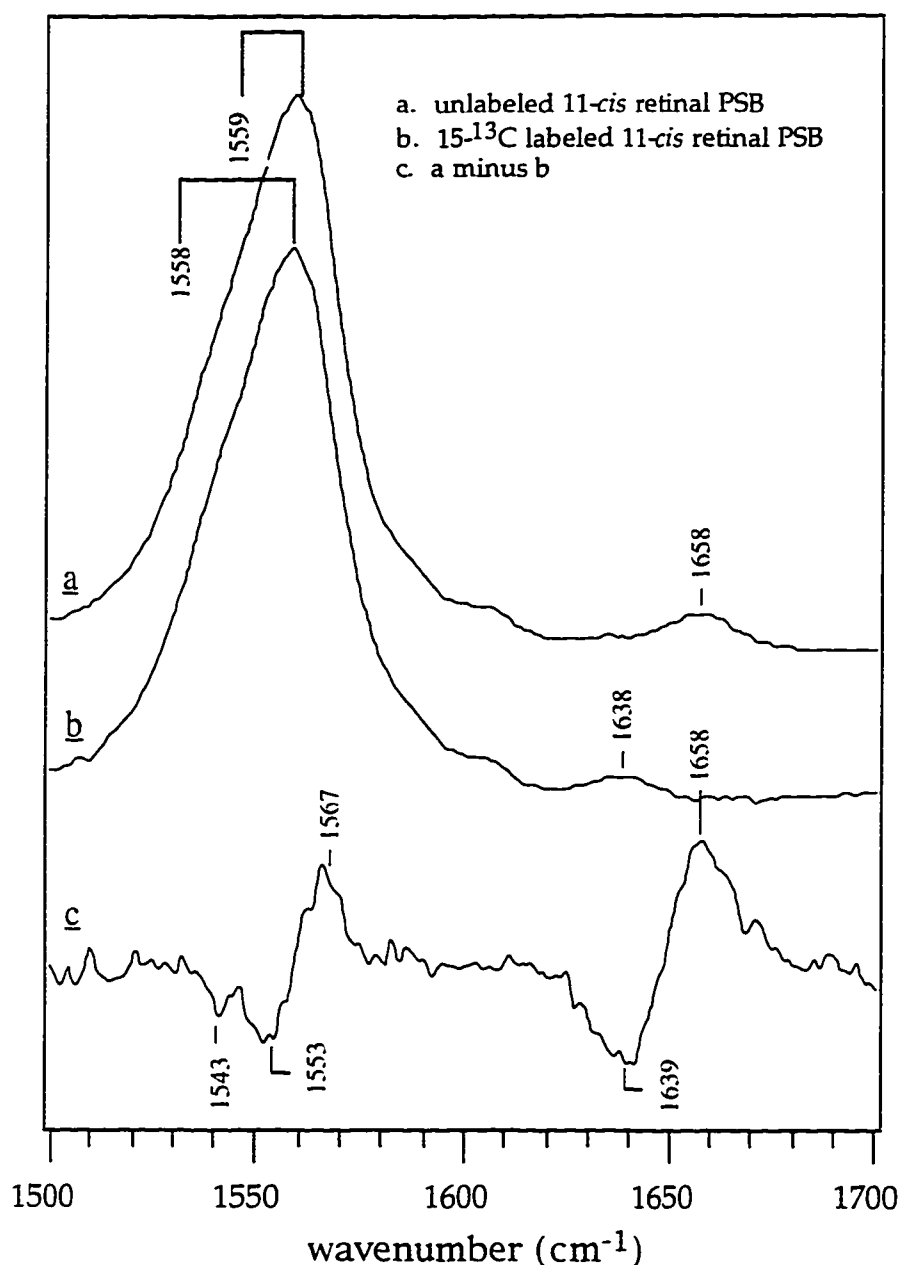


Figure 6.4 a). Pre-resonance Raman spectrum of 11-*cis* retinal protonated Schiff base solublized in methanol in the region of 1500-1700 cm^{-1} . b). pre-resonance Raman spectrum of 11-*cis* retinal protonated Schiff base ^{13}C labeled at the 15 position solublized in methanol in the region of 1500-1700 cm^{-1} . c). difference spectrum resulting from the subtraction of the spectrum in b from the one in a.

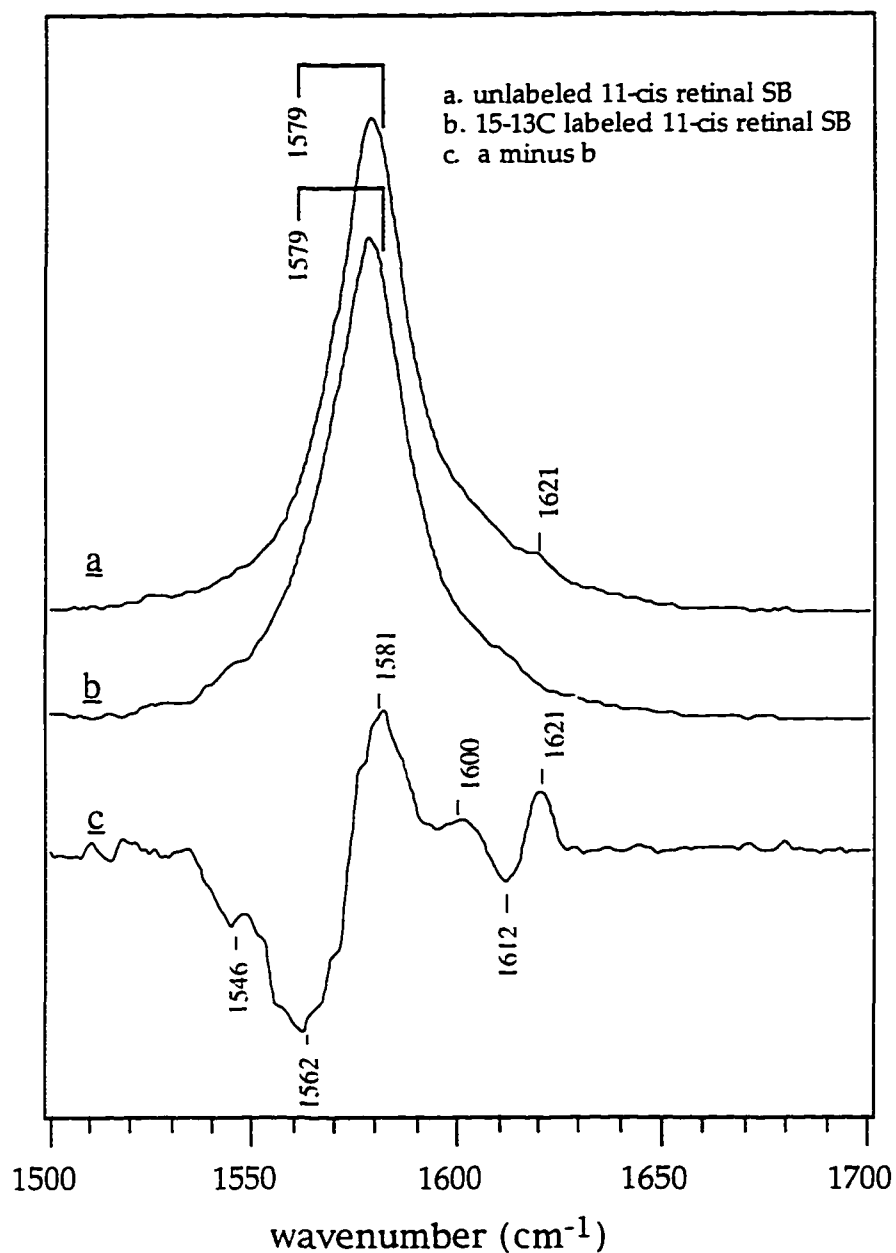


Figure 6.5 a). Pre-resonance Raman spectrum of 11-*cis* retinal Schiff base solubilized in CCl_4 in the region of 1500-1700 cm^{-1} . b). pre-resonance Raman spectrum of 11-*cis* retinal Schiff base ^{13}C labeled at the 15-position solubilized in CCl_4 . 5c). difference spectrum resulting from the subtraction of the spectrum of b from that of a.

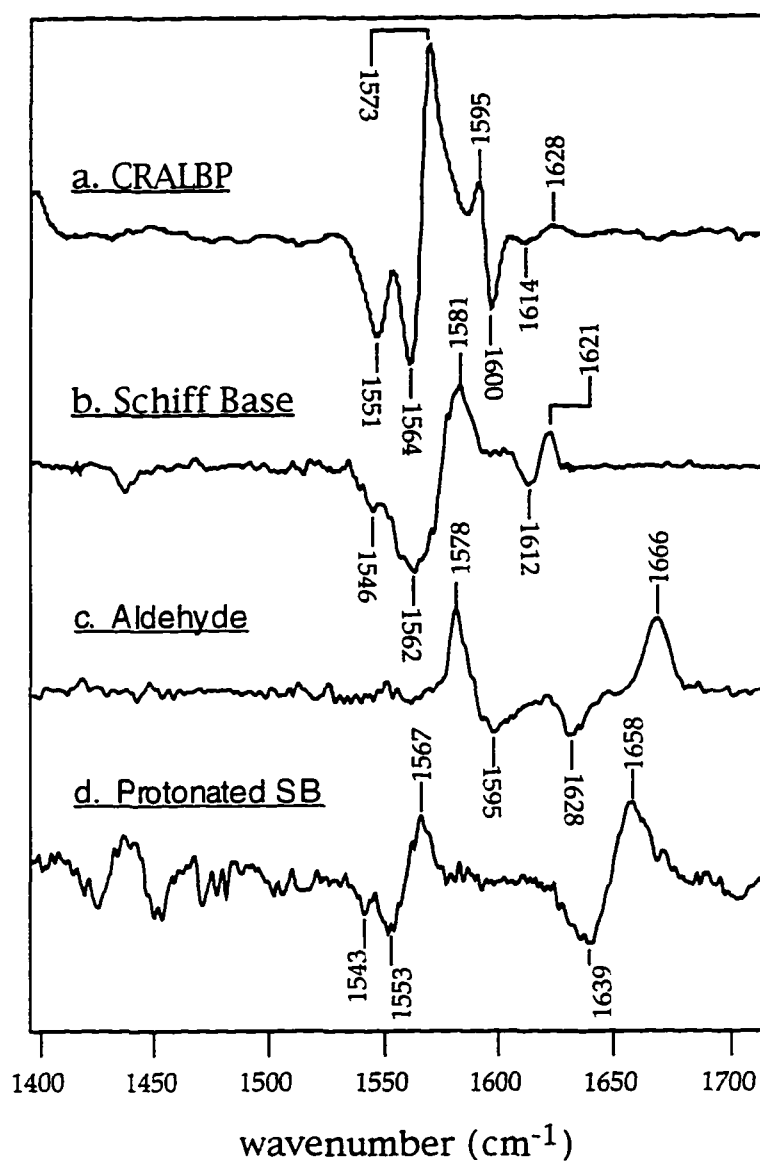


Figure 6.6 Comparison of the Raman difference spectrum in the ethylenic-Schiff Base region (1400 cm^{-1} - 1700 cm^{-1}) of CRALBP (a) to those of the model compounds in CCl_4 solution: Schiff Base (b), Aldehyde (c) and Protonated Schiff Base (d).

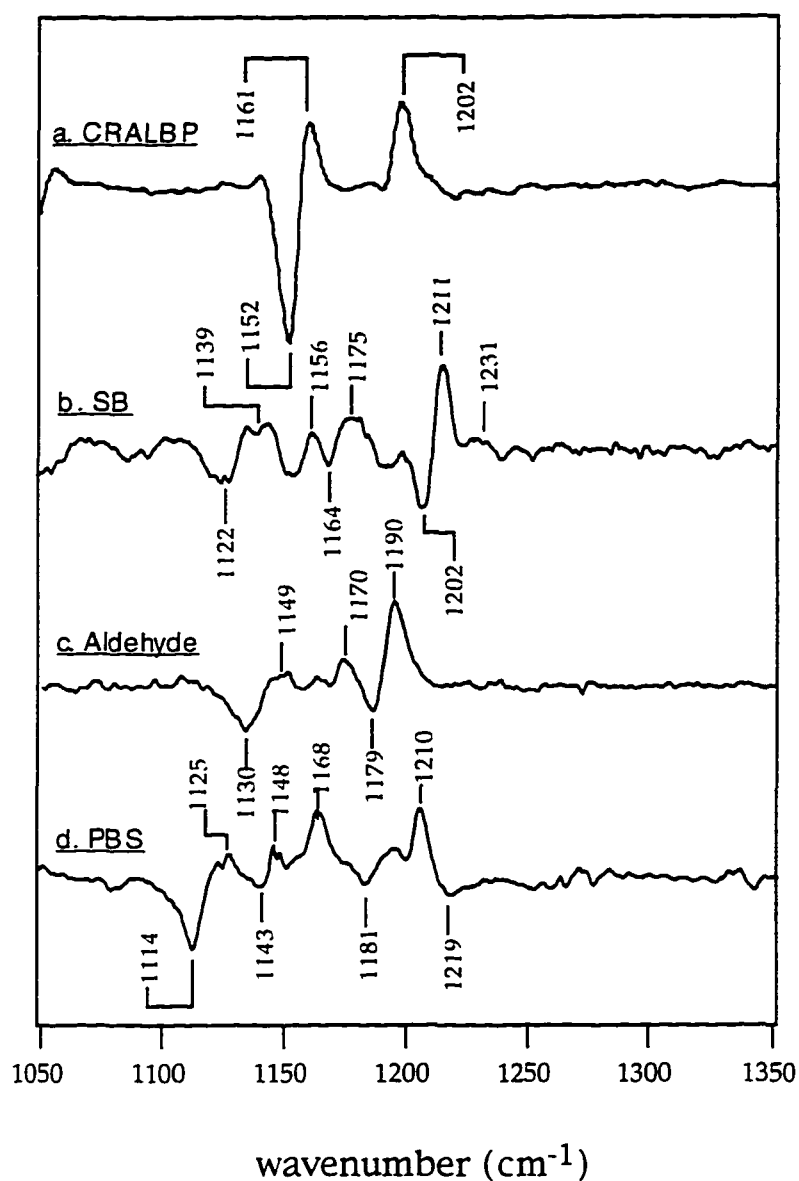


Figure 6.7 Comparison of the Raman difference spectrum of CREALBP (a) in the fingerprint region (1050 - 1350 cm^{-1}) to those of the model compounds in CCl_4 solution: SB (b), Aldehyde (c) and PBS (d). The Raman difference spectra were obtained as described in Materials and Methods.

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