MAGLIOZZO, RICHARD S.

CHEMICAL AND PHYSICAL STUDIES OF TWO BACTERIAL FERREDOXINS

City University of New York

PH.D. 1981

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages _____
2. Colored illustrations, paper or print _____
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages __✓__
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

CHEMICAL and PHYSICAL STUDIES of TWO BACTERIAL FERREDOXINS

by

Richard S. Magliozzo

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

1981

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5/26/81
Date

Chairman of Examining Committee

July 30, 1981
Date

Executive Officer

Supervisory Committee

ABSTRACT

CHEMICAL and PHYSICAL STUDIES of TWO BACTERIAL FERREDOXINS

by

Richard S. Magliozzo

Adviser: Professor William V. Sweeney

The thesis is primarily composed of an investigation of the pH dependence exhibited by the midpoint reduction potential of *Clostridium pasteurianum* 2(4Fe-4S) ferredoxin. The experiments described involve the determination of the $E_{\frac{1}{2}}$ vs. pH behavior in buffer with high ionic strength and an examination of the dependence of nmr, epr and CD spectra on pH. The results of the spectroscopic investigations indicate that the protein does not exhibit a pH dependent conformation equilibrium suggesting that the pH effect arises from oxidation state dependent hydrogen ion equilibria. The data fit a model describing two equivalent sites of protonation per molecule of ferredoxin, each with a pK = 7.4 in the oxidized form and a pK = 8.9 in the reduced form. These proton equilibria may be assigned to the 4Fe-4S centers in this ferredoxin. Such a result is consistent with known properties of iron-sulfur proteins (including hydrogenase) and is likely to be a general feature of the chemistry of iron-sulfur centers. It is suggested that the

involvement of iron-sulfur protein centers in energy conservation at Site I in mitochondrial electron transport may represent a specialization of the observed oxidation state dependent hydrogen ion equilibrium. The thesis also contains preliminary studies of the reconstitution of iron-sulfur centers in *Azotobacter vinelandii* Fd I and in a synthetic tridecapeptide. The details of a synthetic route to a water soluble 4Fe-4S model compound and an attempt to form a CO adduct of ferredoxin are also described. The effect of DMSO and EDTA on *A. vinelandii* Fd I is described. Appendix I is entitled "Study of the Influence of NH···S Hydrogen Bonds on the Reduction Potential in *Clostridium pasteurianum* 2(4Fe-4S) Ferredoxin Using Deuterium Exchange", W.V. Sweeney and R.S. Magliozzo, and appears in Biopolymers 19: 2133 (1980). The experiments described there concern the deuteration of both slowly and rapidly exchanging protons and subsequent measurement of midpoint reduction potentials in the derivatives. The results indicate that hydrogen bonding from amide donors to sulfurs in the iron-sulfur centers does not significantly affect the reduction potential of the ferredoxin.

ACKNOWLEDGEMENTS

I extend sincere thanks to all who contributed to my education as a graduate student, with special appreciation expressed to William V. Sweeney. I am grateful for the freedom with which he shared his knowledge and expertise and especially for the creation of an atmosphere that made it possible for me to express ignorance without embarrassment. His assistance in the preparation of this manuscript and the co-authorship of Appendix I are also acknowledged with appreciation. The members of my thesis committee are also thanked for their contributions and suggestions.

I am grateful for the opportunity to thank several other friends who contributed to the completion of the work described here: Venera Grasso, for the preparation of *Azotobacter vinelandii* ferredoxin I; Michael Pencak, for some very able technical aid; Hugo Schimatz, for the glassblowing requests he filled; Louis F. DiMauro, for helpful discussions concerning spectroscopy and theoretical chemistry; Janis Young, for her gift of synthetic peptides; and my father, for his graphic arts expertise.

I dedicate this work to my parents and to Cathy.

TABLE OF CONTENTS PAGE

LIST OF TABLES PAGE

LIST OF ILLUSTRATIONS

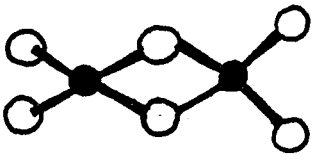
INTRODUCTION

Ferredoxins are low molecular weight, iron-sulfur proteins that have been isolated from bacterial, plant and animal sources (1). They are electron transfer proteins, the redox center of which contains ferrous and ferric iron, sulfide, and cysteinyl sulfur ligands (2). The structure of the iron-sulfur centers in ferredoxins and HiPIP's has been elucidated by x-ray analyses of three proteins and Figure I presents the three presently recognized types. The structure I appears in 2Fe-2S, plant-type ferredoxins, as in spinach and parsley ferredoxin (3,4). The structure II appears in bacterial ferredoxins of three types: clostridial-type, 2(4Fe-4S) ferredoxins as in *Peptococcus aerogenes*; high-potential iron protein, HiPIP, as in *Chromatium vinosum*; and ferredoxin I from *Azotobacter vinelandii* (5-9). The structure III, only recently described by D. Stout (9,10), is also found in ferredoxin I from *A. vinelandii* and is a novel structure. The studies herein described concern the properties of the 2(4Fe-4S) ferredoxin from *Clostridium pasteurianum* and to a lesser extent, the ferredoxin I from *A. vinelandii*. A recent review (11) presents an overview of iron-sulfur proteins containing 4Fe-4S centers.

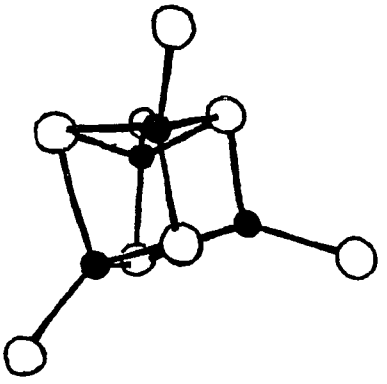
A. *Clostridium pasteurianum* ferredoxin

1. Structure

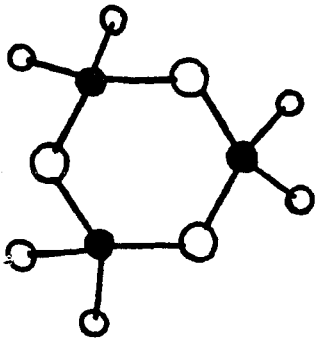
Figure I. The structure of iron-sulfur centers (filled circles are Fe atoms)



I



II



III

The ferredoxin from *C. pasteurianum* was first isolated by Mortenson (12). The amino acid sequence has been determined (13) and its structure may be compared to that of *P. aerogenes* ferredoxin, the structure of which has been determined by x-ray analysis (14). The *P. aerogenes* structure serves as a good model for clostridial-type ferredoxins since the sequence homologies are extensive from species to species. Notably, 39 of the 55 residues are conserved, for example, in the *P. aerogenes* and *C. pasteurianum* ferredoxins and the total number of hydrophobic, neutral, acidic and basic residues is identical in both proteins (27, 20, 7, 1, respectively (15)). There are many other additional characteristics common to all the clostridial-type ferredoxins that allow valid comparisons to the *P. aerogenes* structure: these would include the spectroscopic properties, iron and acid-labile sulfur content, and magnetic properties. (Any discussions of the structure of *C. pasteurianum* ferredoxin contained here are founded upon these similarities and are based on the *P. aerogenes* structure). The ferredoxin contains 2 (4Fe-4S) centers linked to the polypeptide by the sulfhydryl groups on 8 cysteinyl residues. Cysteines 8, 11, 14, and 45 supply thiolate ligands to one cluster and cysteines 18, 35, 38, and 41 supply thiolate ligands to the other, giving an approximate two-fold symmetry axis to the molecule. The iron in the clusters only approximate tetrahe-

dral geometry and the cube-like structure is actually distorted to D_{2d} symmetry. The two 4Fe-4S centers are approximately 12 Å apart. They are buried in hydrophobic regions of polypeptide, though cysteines 11 and 38 are exposed to the solvent (14).

2. Metabolism

The role of ferredoxin in the redox-related metabolism of *C. pasteurianum* has been determined and its importance in glucose metabolism is illustrated in Figure II (2). The reduced ferredoxin also serves to shuttle electrons in the hydrogenase catalysed reaction $Fd_{rd} + 2H^+ \rightarrow H_2 + Fd_{ox}$ (16). It is this couple that is used in the potential measurements discussed in ensuing sections. *C. pastuerianum* ferredoxin is also central in the nitrogenase-linked electron transport reactions (2) as illustrated in Figure III.

3. Spectroscopic characteristics

a. UV-visible

The UV-visible spectrum of oxidized *C. pasteurianum* appears in Figure IV. The notable feature is the broad absorption band at 390 nm (ε = 30.6 $mM^{-1} \cdot cm^{-1}$) due to S→Fe charge

Figure II. The role of ferredoxin in glucose metabolism of *Clostridium pasteurianum*

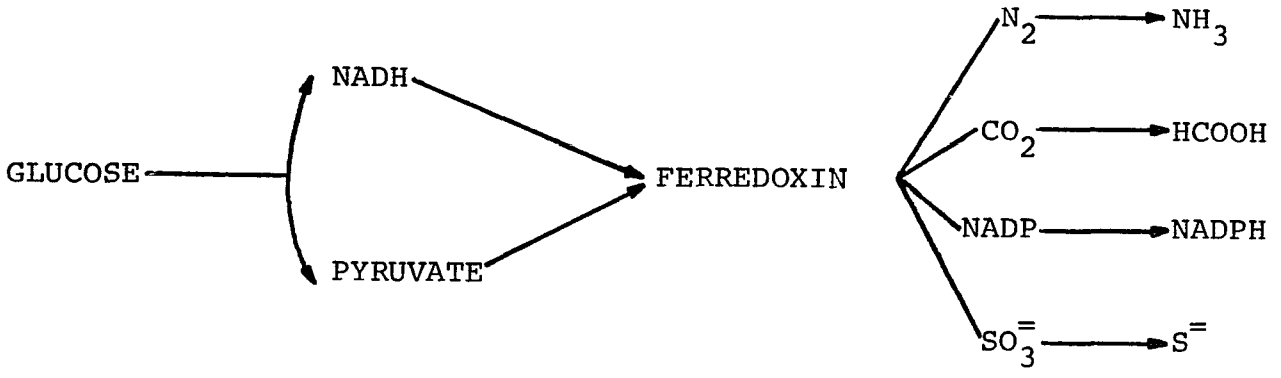

GLUCOSE
NADH
PYRUVATE
FERREDOXIN
N_2
NH_3
CO_2
HCOOH
NADP
NADPH
$SO_3^=$
$S^=$

Figure III. The role of ferredoxin in the nitrogenase linked electron transport of *Clostridium pasteurianum*

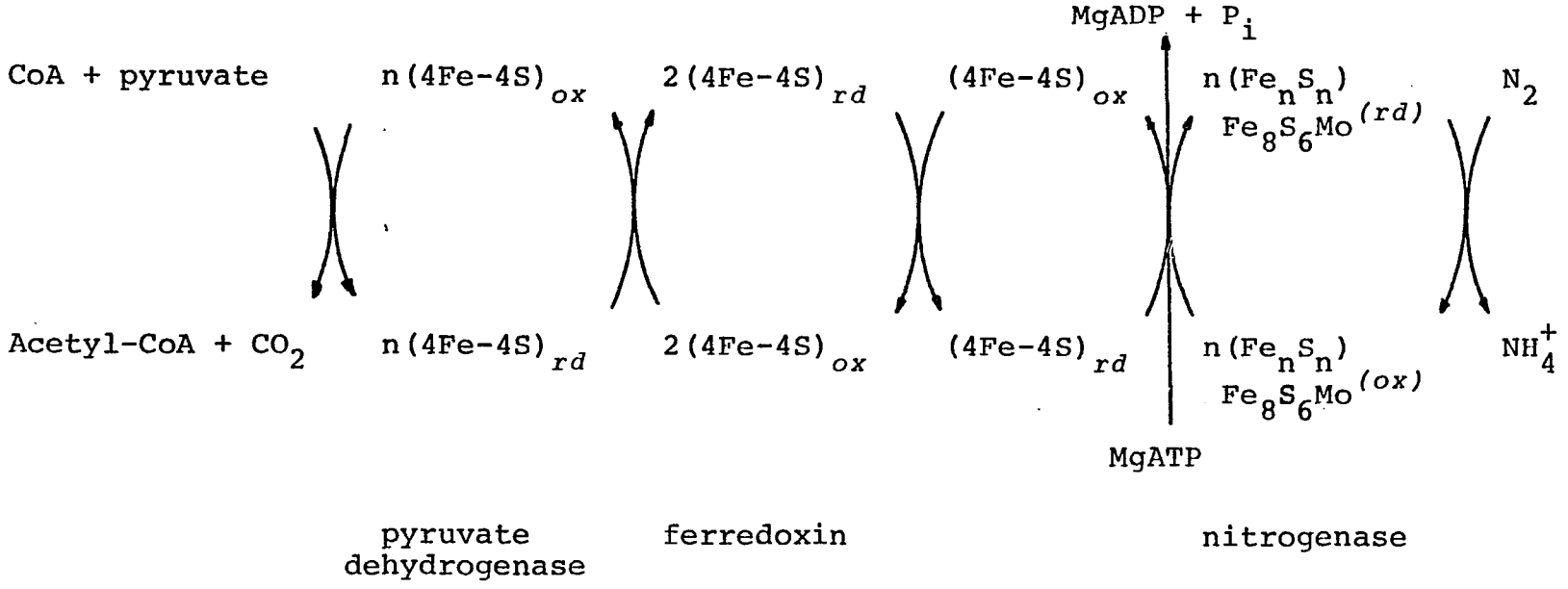
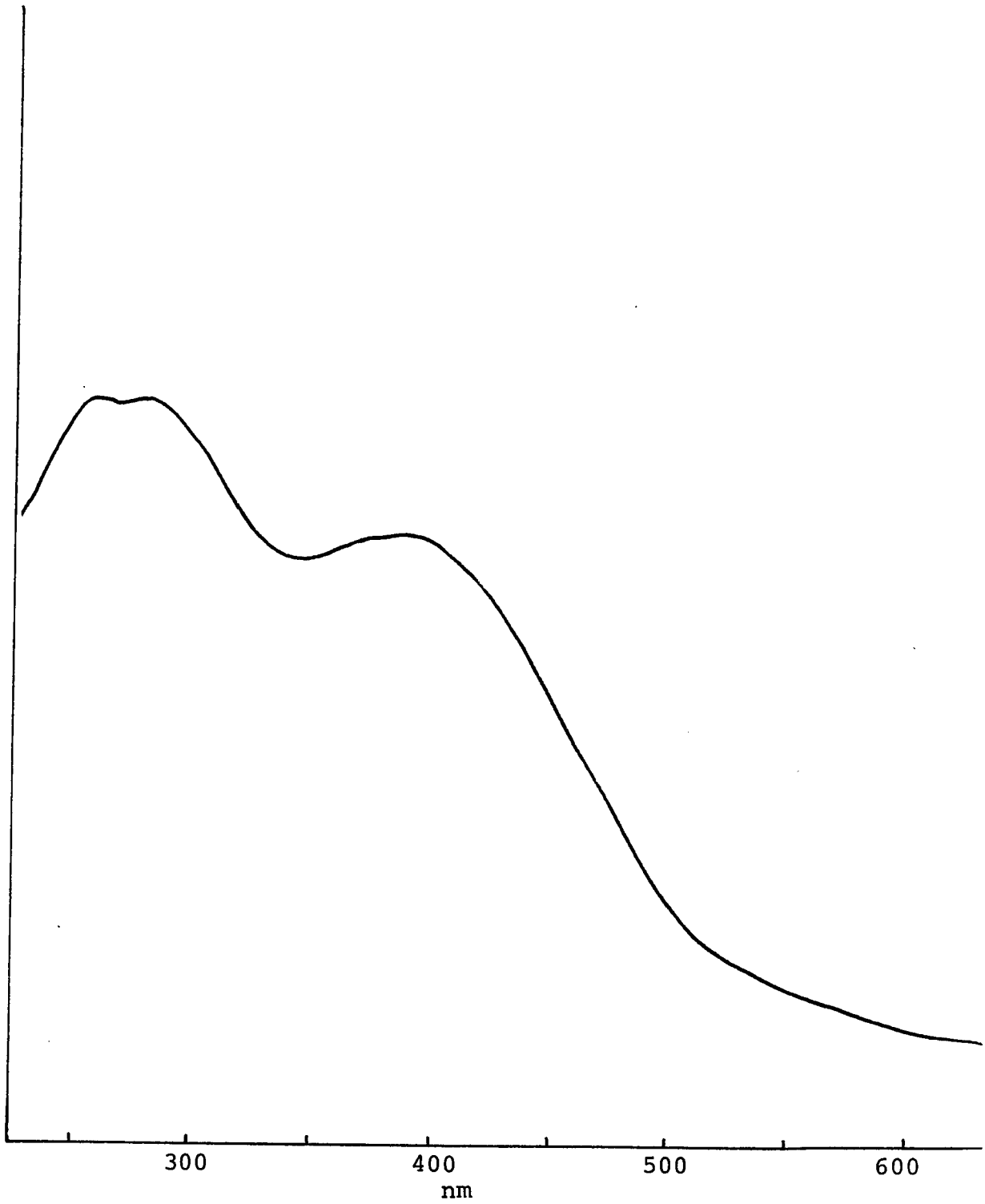

MgADP + P_i
CoA + pyruvate
$n(4Fe\text{-}4S)_{ox}$
$2(4Fe\text{-}4S)_{rd}$
$(4Fe\text{-}4S)_{ox}$
$n(Fe_nS_n)$
$Fe_8S_6Mo^{(rd)}$
N_2
Acetyl-CoA + CO_2
$n(4Fe\text{-}4S)_{rd}$
$2(4Fe\text{-}4S)_{ox}$
$(4Fe\text{-}4S)_{rd}$
$n(Fe_nS_n)$
$Fe_8S_6Mo^{(ox)}$
NH_4^+
MgATP
pyruvate dehydrogenase
ferredoxin
nitrogenase

Figure IV. The optical spectrum of *Clostridium pasteurianum* 2(4Fe-4S) ferredoxin


300
400
500
600
nm

transfer bands (17, 18). The quotient A_{390}/A_{280} is used to assess the purity of this ferredoxin since this 'purity ratio' reflects the amount of chromophore compared to the amount of polypeptide and is a useful guide in any purification procedure. The absorbance at 390 nm is bleached upon reduction of the ferredoxin and the ratio of extinction coefficients at 425 nm (where the greatest difference occurs between the reduced and oxidized absorbances) is 0.435 (19).

b. magnetic

The low temperature (∿ 13 K) epr spectrum of one and two electron reduced *C. pasteurianum* ferredoxin appears in Figure V and VI. This ferredoxin exhibits the $g_{av} = 1.96$ signal typical of reduced 4Fe-4S centers in bacterial-type ferredoxins. The ferredoxin is epr-silent as isolated because of antiferromagnetic exchange coupling between iron atoms in oxidized clusters that results in an S = 0 ground state at low temperature (20). However, each cluster can accept one electron upon chemical or enzymatic reduction (e.g., sodium dithionite or hydrogenase) and the spectrum in Figure VI represents a sample containing approximately 85% of the ferredoxin with both clusters reduced ($2e^-$/ mole). Such spectra contain a superposition of the spectrum arising from one-electron reduced molecules (Figure V) and the

Figure V. The epr spectrum of *Clostridium pasteurianum* ferredoxin (partially reduced sample)

Conditions: temperature, 13 K; gain, 630; modulation amplitude, 2.5 G; power, 2 mW; frequency, ν, 9.40 GHz; buffer, 0.05 mM potassium phosphate (pH 7.5)

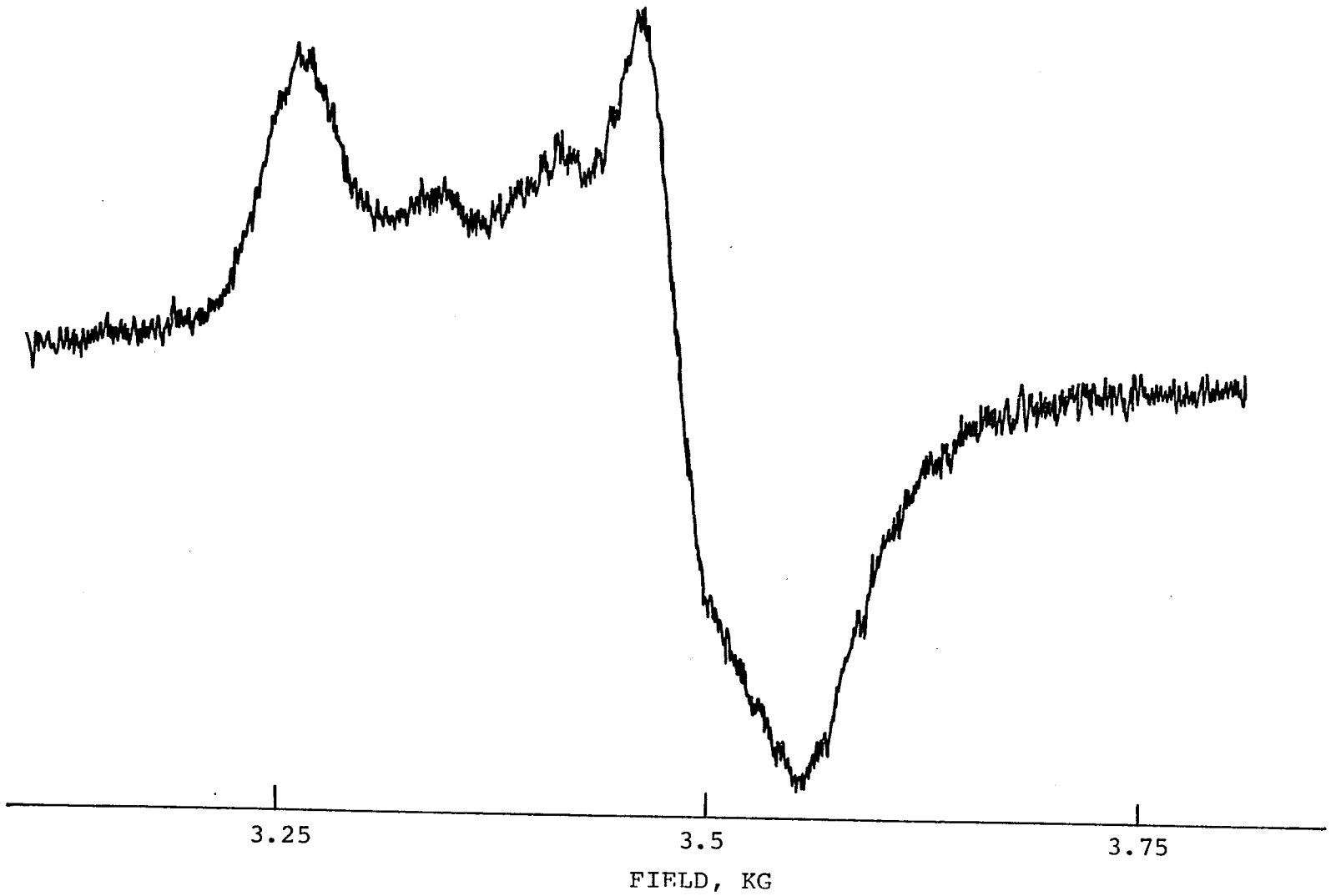
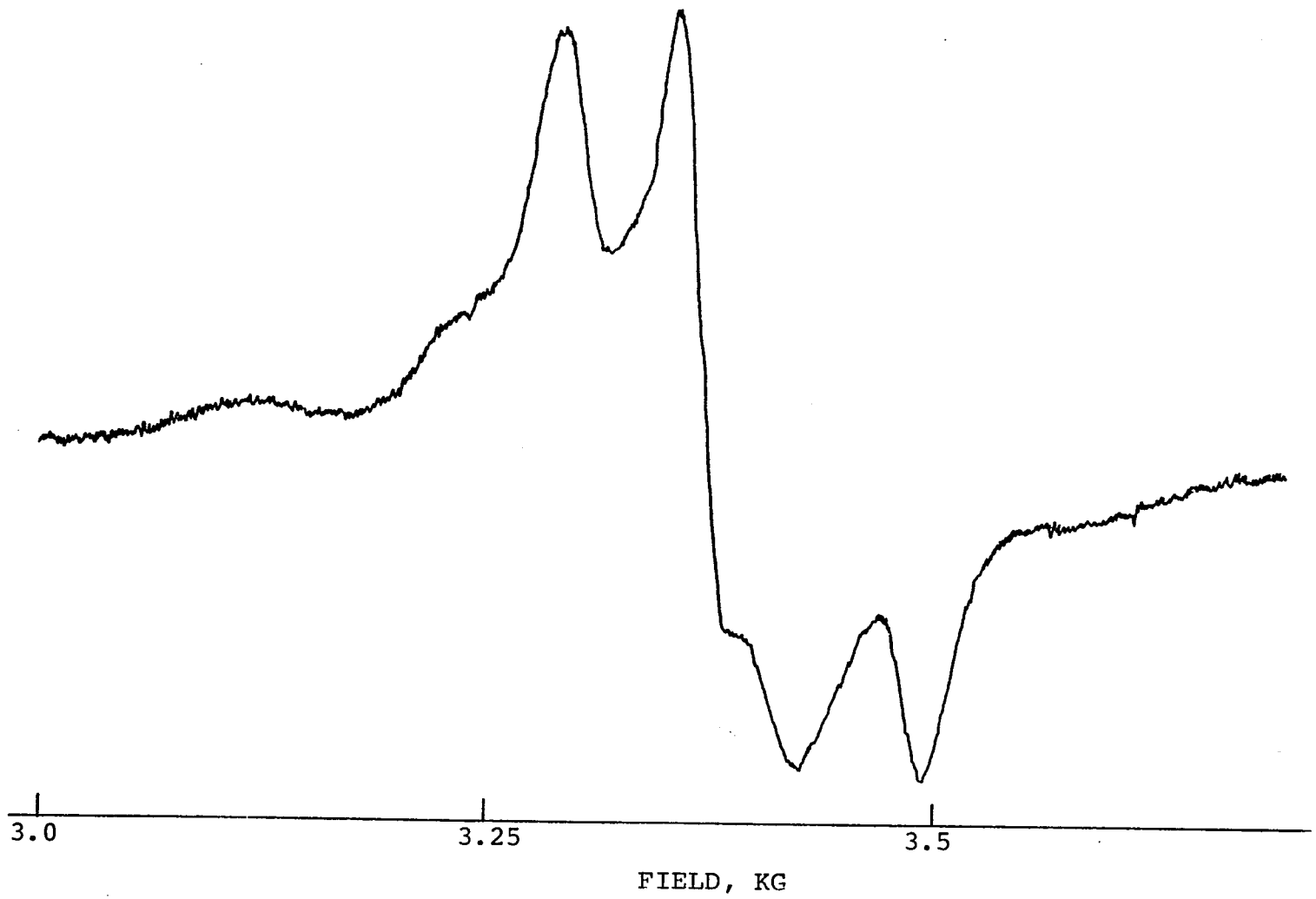

3.25
3.5
3.75
FIELD, KG

Figure VI. The epr spectrum of *Clostridium pasteurianum* ferredoxin (near fully reduced sample)

Conditions: temperature, 12 K; gain, 200; modulation amplitude, 2 G; power, 5 mW; ν, 9.401 GHz; buffer, 0.04 M potassium phosphate in 0.5 M NaCl


3.0
3.25
3.5
FIELD, KG

fully reduced sample spectrum arising from protein molecules in which the two reduced clusters interact.

Epr spectroscopy of *C. pasteurianum* ferredoxin, as it finds application here, is useful as an analytical tool in examining protein conformation. The g-values and linewidths may be compared under different solvent conditions, for example, to probe geometric changes in the paramagnetic centers.

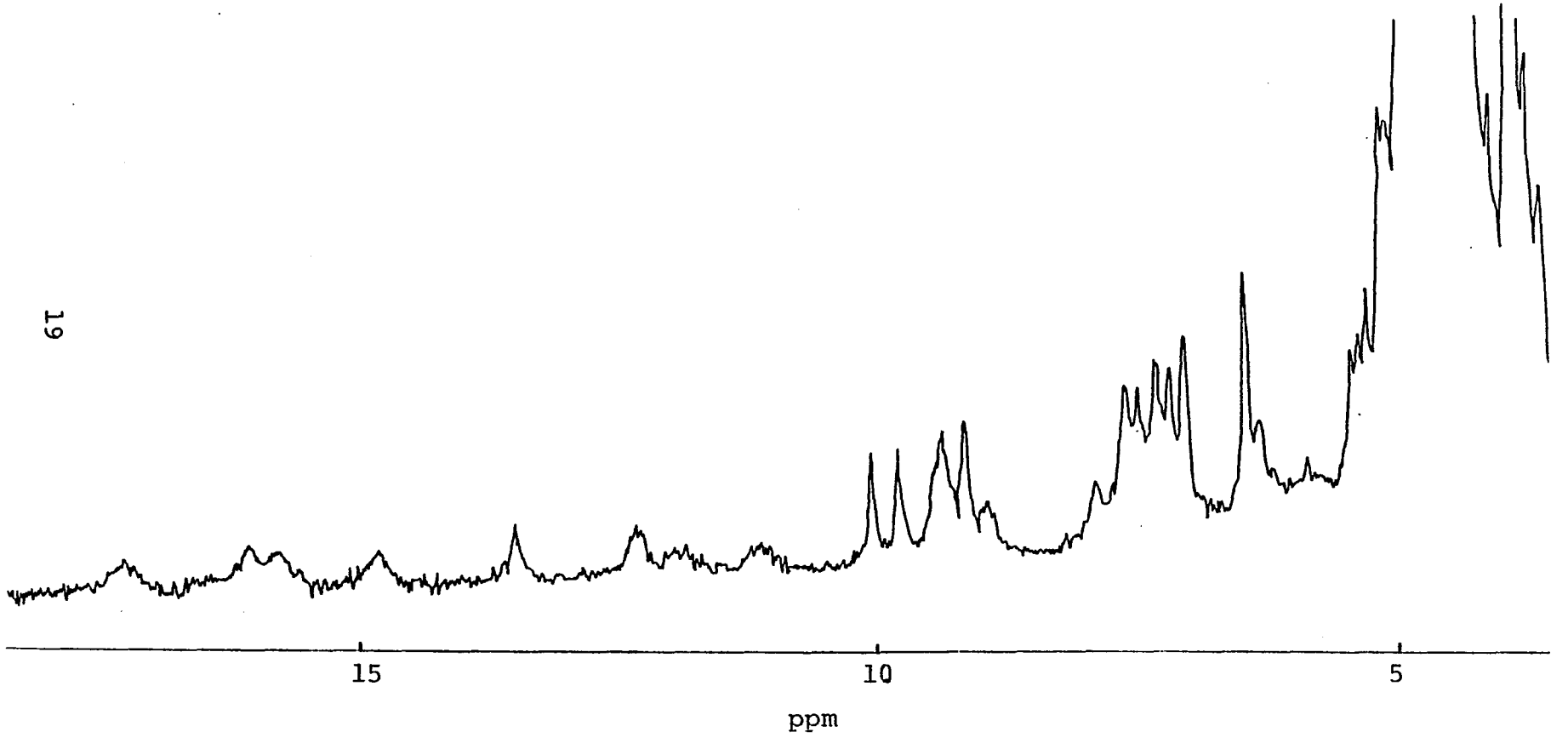
A typical ^{1}H-nmr spectrum of oxidized *C. pasteurianum* ferredoxin appears in Figure VII. The notable features here are the single proton resonances in the ∿10-20 ppm region (from DSS). They arise from β-cysteinyl protons whose resonance frequency is shifted downfield due to the paramagnetism of oxidized clusters at room temperature (21). These resonances serve as good probes of cluster geometry and the application of ^{1}H-nmr spectroscopy here is also a useful tool in monitoring protein conformation.

4. Synthesis and reconstitution of 4Fe-4S centers

The mechanism of *in vivo* synthesis of iron-sulfur centers is not known though the *in vitro* reconstitution of 4Fe-4S centers in *C. pasteurianum* ferredoxin is straightforward. Apoferredoxin (polypeptide lacking Fe-S centers) may be reconstituted to holoprotein by the addition of ferrous iron

Figure VII. The 270 MHz ^{1}H-nmr spectrum of oxidized *Clostridium pasteurianum* ferredoxin

Conditions: temperature, 22°; buffer, 0.04 M potassium phosphate-d, in D_2O (pD = 8.8)


19
15
10
5
ppm

and sulfide in the presence of denaturants and sulfhydryl reducing agents (22). Reconstituted ferredoxin is indistinguishable from native protein and the procedure has application in many of the experiments herein described.

5. Oxidation-reduction characteristics

The 4Fe-4S centers in this ferredoxin function as one electron oxidation-reduction centers and operate between the +1 and +2 states. The 'three state hypothesis' (23) serves to catalogue the oxidation states of 4Fe-4S centers for ferredoxins and HiPIP's in the following way: the "C" state corresponds to the paired spin state for clusters in oxidized or "as isolated" *C. pasteurianum* ferredoxin and the 4Fe-4S center in high-potential iron proteins. The "C^{+}" state is the epr active state in HiPIP type proteins. The "C^{-}" is another paramagnetic state corresponding to reduced *C. pasteurianum* ferredoxin. (Recent iron-sulfur protein literature makes use of the designations +1, +2, and +3 for oxidation states of 4Fe-4S centers, corresponding to the C^{-}, C, and C^{+} states in the 'three state hypothesis'. In some instances, the designations -1, -2, and -3 are used, which correspond to the C^{+}, C and C^{-} states. If a cluster is identified as a 4Fe-4S center, the +1, +2, +3 (or C designations) may be used. However, a cluster formulation which includes the 4

additional Fe-S ligands, must be described by the -1, -2, -3 formal oxidation numbers (or simply the C-state labels) since the formal charge of the thiolate ligands included in a formula such as $Fe_4S_4(SR)_4$ must then be included in the formal oxidation state of the cluster). Thus, formally, the irons may be considered to be 3 ferrous and 1 ferric in the reduced state and 2 ferrous, 2 ferric in the oxidized state, with the inorganic sulfur as sulfide.

Though the formal charge on the clusters can be calculated by assigning oxidation numbers to the iron and sulfur atoms, the electron density in the centers is delocalized and the actual oxidation state of individual irons cannot be assigned even on the time scale of Mössbauer spectroscopy. For example, the average valence assigned to the iron atoms in reduced *C. pasteurianum* ferredoxin is +2.25 (24).

B. *Azotobacter vinelandii* ferredoxin I

The iron-sulfur protein, ferredoxin I, from *Azotobacter vinelandii* is a novel bacterial ferredoxin. Until recently, it was considered a 2(4Fe-4S) protein with unusual redox chemistry because it contains a reducible, low-potential center ($E_{\frac{1}{2}}$ = -420 mv) and an oxidizable, HiPIP-like center ($E_{\frac{1}{2}}$ = + 320 mv) though epr characteristics suggested both clusters operated between the HiPIP oxidation states

(8). Preliminary x-ray data (9) as well as cluster extrusion experiments (25) suggested that Fd I contained one 4Fe-4S center and a second center containing less iron, presumably a 2 Fe-2S center. Most recently, the 2.5 Å resolved x-ray studies completed by D. Stout (10) have elucidated the structure of the unusual center, which contains 3Fe-3S (see Figure I). The structure is consistent with Mössbauer results that indicated the presence of a 3Fe center. It has also been shown that the high potential center is the 4Fe-4S, HiPIP-like center (25). In the absence of the advancement provided by these studies, the problem of identifying clusters in this protein was attempted and a reconstitution experiment is described in later sections.

C. Synthetic analogues

The synthesis of an inorganic model for a ferredoxin iron-sulfur center was first reported by Herskovitz (26). Many models have been synthesized and characterized since that time and have provided simpler routes to descriptions of cluster chemistry and redox properties. A comprehensive review of these appears in Volume III of *Iron-Sulfur Proteins* (27). Included in the list of numerous models described in the literature are several that have small, cysteine-containing synthetic peptides incorporated around 4Fe-

4S centers (28). These 4Fe-4S models have midpoint reduction potentials close to those for protein-bound clusters (e.g., Fe_4S_4(12-peptide)$^{-2}$, 12-peptide = t-BOC-gly-cys-gly-gly-cys-gly-gly-cys-gly-gly-cys-gly-NH_2, $E_{\frac{1}{2}}$ = -0.80v vs. SCE (28)). A description of the reconstitution of a synthetic, cysteine containing tridecapeptide appears in section IV. This experiment is directly related to the cluster replacement experiments in which the 12-peptide is incorporated around a preformed Fe_4S_4 core through the demonstration that short peptides with cys-X-X-cys sequences can accommodate 4Fe-4S centers.

Section III contains a description of the synthesis of a water soluble model compound.

D. *Clostridium pasteurianum* hydrogenase

The hydrogenase from *C. pasteurianum* is a bi-directional iron-sulfur enzyme which catalyzes the following reaction: $H^+ + e^- \rightleftarrows \frac{1}{2}H_2$ (16). It has a molecular weight of 60,000 (29, 30, 31) and there is some disagreement on the number of 4Fe-4S centers it contains: Erbes, et al., (32) reported one center per mole; Chen and Mortenson (33) report 3 centers per mole. The enzyme couples with ferredoxin in solution and crude preparations of hydrogenase (see section II, C) were used in the determination of midpoint

reduction potentials described throughout this work.

GENERAL METHODS

A. Growth of *Clostridium pasteurianum*

Clostridium pasteurianum cultures were started from lyophilized cells using a potato medium (34). Fully grown potato tubes were used as inoculum for 125 ml of synthetic medium in 250 ml volumetric flasks. Traces of $Na_2S_2O_4$ were added to flasks just prior to inoculation and anaerobic plugs (using alkaline pyrogallol or 'Chromosorb' solutions) were used throughout. Flasks were stoppered with anaerobic plugs while still hot from autoclaving and allowed to cool. Cultures were worked up to grow required amounts of cells for either ferredoxin preparation or hydrogenase preparation, using a 10% inoculum for each transfer. Hydrogenase preparation required approximately 5 liters of active culture (yield ≈ 10g/liter wet cells). Ferredoxin preparations required approximately 500 g cells. Large amounts of cells were grown in a Fermatron-150 fermentor from which yields averaged 650g/90 liter run. The following recipe was used for 90 liters of medium (adapted from (34)):

Sucrose (Domino Sugar)	1800g
$MgCl_2 \cdot 6H_2O$	13.3g

NaCl	9g
$Na_2MoO_4 \cdot 2H_2O$ (10% w/v)	9ml
Biotin + p-aminobenzoic acid (solution 0.01% in each)	4.5ml
Na_2SO_4	6.4g
NH_4Cl	144g
$FeCl_3$ (5% in absolute ethanol)	360ml
K_2HPO_4 (in a sterile solution)	400g

The fermentor vessel was autoclaved for 45 minutes with all components in solution except $FeCl_3$ and K_2HPO_4 which were added just prior to inoculation. The pH was adjusted to a value between 7 and 8 with NaOH. Approximately 2 hours before inoculation, oxygen-free nitrogen (or prepurified grade nitrogen scrubbed free of O_2 with alkaline pyrogallol) was vigorously bubbled through the medium. Inoculum (approximately 14 liters active culture) was added during vigorous bubbling of N_2. Gas flow was reduced to a slow rate after one hour and maintained during the entire growth cycle. The pH of the medium was maintained with a pH-stat feature on the Fermatron-150, using 10 N NaOH. Large amounts of base were required due to the production of butyric acid by *c. pasteurianum*. Cells were usually harvested 12-18 hours after inoculation or when Klett turbidimetric readings had reached approximately 450 (green filter). Ten liters of

active culture were saved under nitrogen as inoculum for a second or third growth cycle before which fresh medium was sterilized as described above.

Cells were harvested using a CEPA cell harvester and were stored at 0^{o}. For hydrogenase preparations, cells were harvested from flask cultures using a Sorvall centrifuge.

B. Preparation of *C. pasteurianum* ferredoxin

The 2(4Fe-4S) ferredoxin from *C. pasteurianum* was isolated according to the following procedure, based on the procedure of Rabinowitz (34).

Approximately 1200 g frozen cells were defrosted and suspended in 1200 ml 0.05 M potassium phosphate buffer, pH 7.4, and sonicated in batches for approximately 6 minutes at 0^{o}. The sonicate was centrifuged for one hour at 14,000x g and the supernatant was applied to a DEAE column (300 ml) pre-equilibrated with 0.01 M Tris-HCl (pH 7.4). The protein was washed with 500 ml 0.01 M Tris-HCl (7.4), then 250 ml 0.15 M Tris-HCl (7.4) and finally 250 ml 0.1 M Tris-HCl containing 0.1 M NaCl (pH adjusted to 7.4 after addition of NaCl). The ferredoxin was eluted with a linear gradient composed of 300 ml 0.1 M Tris-HCl/ 0.1 M NaCl and 300 ml 0.1 M Tris-HCl/ 0.5 M NaCl, both at pH 7.4.

The ferredoxin containing fractions from this first chromatography were diluted approximately 4 times with water and applied to a second DEAE column (300 ml) pre-equilibrated with 0.1 M Tris-HCl in 0.1 M NaCl (pH 7.4). The column was washed with 250 ml 0.1 M Tris-HCl/ 0.1 M NaCl (pH 7.4), 0.1 M Tris-HCl/ 0.16 M NaCl (pH 7.4) and the protein was eluted with a linear gradient composed of 0.1 M Tris-HCl/0.16 M NaCl and 0.1 M Tris-HCl/0.5 M NaCl (pH 7.4, 300ml each). Ferredoxin-containing fractions from the second chromatography were concentrated by ultrafiltration to a concentration of approximately 1 mg/ml. The solution was made 60% saturated in ammonium sulfate under a blanket of nitrogen. The 60% 'cut' was centrifuged at 10,000 x g for 30 minutes. The supernatant was then made 90% saturated in ammonium sulfate and the ferredoxin was recentrifuged. The purity ratio of the ferredoxin at this stage of the preparation was usually near 0.7 (Ratio = A_{390}/A_{280}). The precipitated ferredoxin was washed once with saturated ammonium sulfate and the pellet was dissolved in 0.1 M Tris-HCl (pH 7.4 at 5^{o}) containing 0.1 M NaCl. Approximately 60 mg portions were applied to a Sephadex G-75 column (5 x 100 cm, refrigerated at 5^{o}). Fractions with a purity ratio of 0.80 were pooled, precipitated in 90% saturated ammonium sulfate, and stored anaerobically at 5^{o}.

C. Preparation of *C. pasteurianum* hydrogenase

The following procedure was used to prepare partially purified hydrogenase which was used for midpoint reduction potential measurements (19).

Forty to sixty grams of freshly harvested cell (taken from very active cultures that exhibited vigorous evolution of H_2) were suspended in 40-60 ml of 0.1 M potassium phosphate buffer (pH 7.4) that had been saturated with hydrogen and then made approximately 0.01 M in $Na_2S_2O_4$. The cell suspension was sonicated at 0^o (Sonifier Cell Disruptor, Model W140, Heat Systems Ultrasonics) at approximately 80% of full power. The sonicate was centrifuged at 14,000 x g for one hour and the supernatant was loaded onto a DEAE column. The column had been equilibrated first with 0.01 M potassium phosphate (pH 7.4), then with 4 column volumes of de-aired (H_2) 0.1 M potassium phosphate and finally with approximately 2 column volumes 0.1 M potassium phosphate made 0.01 M in $Na_2S_2O_4$. The green 'pass thru' was collected under hydrogen in 15 ml centrifuge tubes. Peak fractions were treated at 60^o for 10 minutes and centrifuged at 3000 x g for 45 minutes with serum caps on the tubes. The pale green supernatant was transferred to sample tubes by syringe, under hydrogen gas. Sample tubes of approximately 0.5 ml were prepared from disposable pipettes which were cut

and sealed with serum caps. Samples were quickly frozen in liquid nitrogen and stored in a liquid nitrogen dewar.

D. Preparation of nmr samples

Most nmr spectra were recorded on solutions which were 100% D_2O and samples were generally prepared in the following manner:

Ferredoxin, usually recovered from 90% saturated ammonium sulfate, was dissolved in 0.1 M K_2DPO_4/KD_2PO_4 buffer prepared by titrating a 0.1 M solution of KD_2PO_4 with 40% KOD to the desired pD. ($pD = pH_{(meter\ reading)} + 0.41$ (35)). This solution was placed in an AMICON ultrafiltration cell (YM 5 membrane) and concentrated to approximately 250 microliters. The concentrate was diluted to approximately 3 ml with the same buffer and reconcentrated. The second solution was diluted again and finally concentrated to 250 microliters to remove the last traces of water. To the final concentrated solution (containing approximately 5 mg ferredoxin) was added 25 microliters 1% DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) in D_2O. Samples were generally loaded into Wilmad cylindrical microtubes, 508-CP which contain a 140 µl well.

E. Preparation of epr samples

Fully reduced samples were prepared by de-airing a solution of ferredoxin (usually 0.5 mM) with nitrogen and transferring this solution anaerobically to a sample tube. A 20 to 30-fold excess of sodium dithionite in buffer was then added under nitrogen or argon. The bleaching of brown color was immediate under these conditions and samples were frozen in liquid nitrogen immediately after addition of reductant. For partially reduced samples, a 20 to 40-fold excess of $Na_2S_2O_4$ in buffer was added to the protein solution in the epr tube without de-airing. Samples were then frozen in liquid nitrogen. In some cases, the degree of reduction was adjusted during the recording of epr spectra by removing a sample from the spectrometer, plunging the tube into water to thaw the sample and opening the tube to the atmosphere momentarily. The sample was then agitated and re-frozen.

F. Procedure for measurement of midpoint reduction potentials

All ferredoxin reduction potential measurements reported here were performed in the following manner (19): a buffered solution of ferredoxin was bubbled for 20 minutes with H_2 using needles, in a 3 ml cuvette fitted with a serum cap. The gas (prepurified grade) was scrubbed by bubbling it through 15% pyrogallic acid in 50% KOH, then bubbling through

water to prevent contamination of solutions with pyrogallol aspirated from the bubbler. The absorbance at 425 nm of the ferredoxin solution was then recorded. A sample of hydrogenase (Section II, C) was defrosted under H_2 and 6 microliters were added to the ferredoxin solution by syringe. The absorbance at 425 nm was then monitored until it no longer decreased. The final absorbance was then recorded, A_{rd}. The ferredoxin solution was then bubbled for approximately 20 minutes with N_2 (prepurified grade) which contains enough oxygen to gently re-oxidize the ferredoxin. The nitrogen in this step was first bubbled through water to saturate the gas with water vapor in order not to concentrate the ferredoxin solution during bubbling. The absorbance at 425 nm was recorded again. The solution was bubbled again with nitrogen for 10 minutes and the absorbance re-measured. This process was repeated until a constant absorbance was achieved, to insure that the ferredoxin was completely re-oxidized. The re-oxidized absorbance at 425 nm was usually greater than 95% of the original, oxidized absorbance. If 95% of the original absorbance was not recovered, the data were discarded since the loss of protein may occur in the reduced state, or upon re-oxidation and no proper correction can be made. The ratio of oxidized to reduced protein is calculated from the following:

$$\frac{A_{rd} - 0.435(A_{ox})}{A_{ox} - A_{rd}}$$

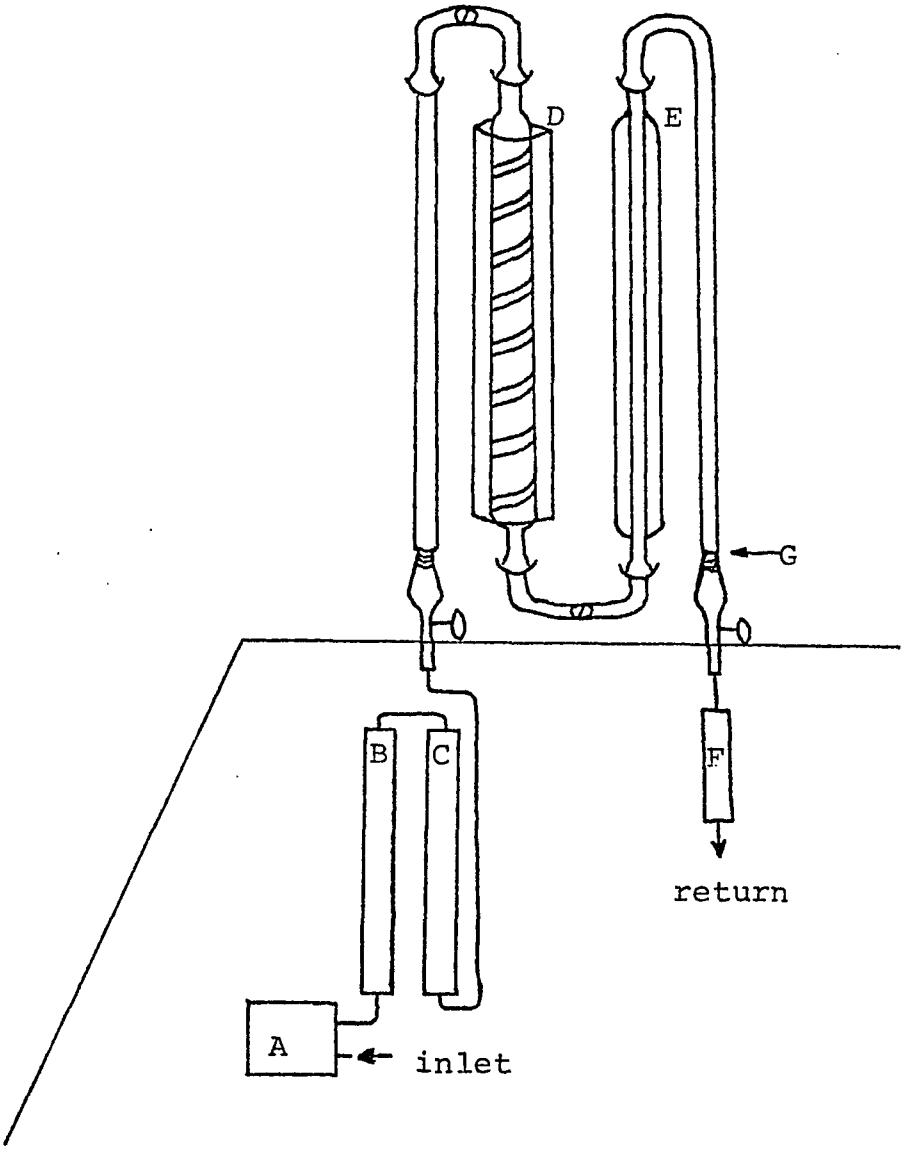
The value 0.435 is the ratio of extinction coefficients at 425 nm for the reduced/oxidized *C. pasteurianum* ferredoxin (19). The pH of the ferredoxin solution was carefully measured after completion of the reduction procedure (Radiometer pH meter 26). The Nernst equation is applied (n = 1) with the solution potential equal to the hydrogen potential. Most measurements were performed at one atmosphere giving $E_{sol'n} = -59(pH)$ at 25^{o}.

G. Preparation of glove box

A glove box was prepared as required according to the following procedure. All solutions required in the box were thoroughly degassed before placing them in the box. The box was pumped out for approximately 30 seconds using the house vacuum or a mechanical pump and purged with oxygen-free N_2 through 4 cycles. It was then filled with nitrogen which was continuously circulated through molecular sieves and an oxygen scavenger (BASF R3-11). Figure VIII illustrates the arrangement of the gas train. A slow purge of N_2 was maintained during use of the box. Gas was circulated using a small diaphragm pump. The box was thoroughly purged for 24 hours with the glove ports covered with aluminum foil before

Figure VIII. Sketch of gas train used for re-cycling and purifying glove box atmosphere

A, diaphragm pump; B, molecular sieves Grade 513; C, molecular sieves Grade 626 Binderless; D, BASF catalyst; E, condenser loaded with Grade 513 sieves; G, KOVAR seals; F, molecular sieves Grade 513


D
E
G
B
C
F
return
A
inlet

the recirculating system was opened. All transfers into the box were done through a port which was thoroughly purged, through several pump-out cycles. The outlet of the glove box was fed through a bubbler with a float-check valve, filled with oil. Minimum lengths of rubber tubing were used wherever needed for connections to reduce diffusion of air. The catalyst column was attached to a board fitted to the roof of the glove box. Connections from the glass columns to the box were made by Kovar to glass seals. The Kovar sections were silver soldered to the outlet valves on the roof of the box. Joints on the glassware of the purifying train were spherical type with O-rings. The BASF catalyst was reduced before use by slowly passing nitrogen through the column while heating to approximately 130^{o}. Carbon monoxide was added to the nitrogen at a rate such that the temperature of the catalyst reached approximately 190^{o}. Reduction was completed with undiluted CO. The catalyst turns from green to black during the reduction process ($CuO + CO \rightarrow Cu + CO_2$). The temperature of the bed was monitored with a small thermometer attached to a glass hook located on the inner wall of the column. The column was wrapped with heating tape and was air jacketed. Gas was passed from the top to the bottom of the bed and the efflux was cooled through a condenser filled with molecular sieves (Davison, Grade 513) to trap H_2O released during reduction. The gas pumped through the

catalyst from the glove box was passed through Grade 513 and Grade 626 Binderless sieves (to trap H_2S and mercaptans) before entry into the catalyst column.

Study of the pH Dependence of the Midpoint Reduction Potential in *Clostridium pasteurianum* ferredoxin

A. Introduction

Many iron-sulfur proteins exhibit pH dependent midpoint reduction potentials and some examples of these are listed in Table I. Though such dependencies have been known for years, the question of the origin of the effect has not been addressed in the literature. It appears that oxidation state dependent hydrogen ion binding to iron-sulfur centers is a feature of the chemistry of Fe-S centers in general, in view of the following: Hydrogenase is an iron-sulfur protein; iron-sulfur proteins are implicated in the proton translocating property exhibited by energy transducing membranes (39, 40); and the observation that the slope of $E_{\frac{1}{2}}$ vs. pH has a negative value for all the proteins (except Hi-PIP) listed in Table I, despite varied polypeptide compositions. To test for the existence of oxidation state dependent hydrogen ion equilibria, the pH dependence of the midpoint reduction potential of *Clostridium pasteurianum* has been examined. This ferredoxin was chosen because it has no amino acid residue with an intrinsic pK between 6 and 9 and exhibits a significant and well-defined pH dependence.

Table I. The pH dependencies of the midpoint reduction potentials for several iron-sulfur proteins

Cluster Type	Source	Estimated Dependence	pH Range	
2Fe-2S	Spinach	-4 mv/pH unit	7.0-8.2	(36)
	Pseudomonas putida	-30	7.5-?	(37)
	Parsley	-7	6.0-9.0	(37)
2Fe-2Se	Parsley	-14	6.5-8.0	(37)
4Fe-4S	*Peptococcus aerogenes*	-8	6.9-7.6	(36)
	Clostridium pasteurianum	-16	6.6-7.5	(19)
		-12	6.7-7.7	(36)
	Clostridium acidi-urici	-13	6.5-8.2	(19)
		-2	7.0-7.9	(36)
	Clostridium tartarivorum	-3	7.0-7.9	(36)
	Bacillus polymyxa (I)	-11	6.6-7.7	(36)
	Bacillus polymyxa (II)	-24	6.9-7.7	(36)
	Chromatium vinosum	-11	8.1-8.9	(36)
	Chromatium vinosum (HiPIP)	0	7.0-11.0	(38)
2Fe-2S	Ox heart mitochondria, center N-1a	-60	6.2-8.6	(39)
4Fe-4S	Ox heart mitochondria, center N-2	-60	6.8-8.6	(39)

B. Materials and Methods

Cells were grown and ferredoxin isolated according to the procedures in sections II, A and B. Reduction potentials were determined according to the procedure described in section II, F. Ferredoxin solutions were prepared from a highly concentrated stock solution by dilution into the buffer of desired pH. The stock solution was prepared using an AMICON ultrafiltration cell (YM 5 membrane) and distilled water to which a few drops of Tris-HCl buffer (pH 7.4) had been added to adjust the pH to near neutrality. All solutions were prepared to be approximately 0.03 mM ferredoxin by dilution of 30 microliters of stock solution to 3 ml with 0.1 M Tris-Acetate-Glycine-Phosphate buffer containing 0.5 M NaCl. This buffer system was chosen to provide the buffering capacity needed throughout the range of $E_{\frac{1}{2}}$/ pH measurements and to eliminate any effect arising from differences in ionic strength between buffers of different pH. The pH of re-oxidized ferredoxin solutions was used in the calculations because small changes in pH occurred during the reduction procedure. The changes cannot be ascribed to the reduction or re-oxidation parts of the procedure and were small enough that no significant error is introduced into $E_{\frac{1}{2}}$ determinations by omitting such an assignment. (The largest change observed was approximately 0.05 pH units be-

tween the starting buffer and the pH of the solution of re-oxidized ferredoxin {see General Methods section II, F, for other details of the reduction procedure}). The pD of buffers used for nmr samples was calculated by adding 0.4 to the pH read on a Radiometer pH Meter 26 (35). Optical measurements were made using a Cary 219 UV-visible spectrophotometer. Proton nmr spectra were recorded on a Bruker 270 MHz spectrometer at the NIH Regional Facility in New Haven, CT. Epr spectra were obtained using a Varian V-4500 X-band spectrometer equipped with a Heli-Tran liquid helium transfer system (Air Products). Circular dichroism spectra were recorded on a Jasco J-20 spectropolarimeter.

C. Results and Discussion

The pH dependence of the midpoint reduction potential, $E_{\frac{1}{2}}$, for *C. pasteurianum* ferredoxin was determined from pH 6.2 to pH 8.9. At pH values lower than 7.4, $E_{\frac{1}{2}}$ exhibits a dependence of -16mv/ pH unit and at pH values greater than 7.4, a dependence of -30mv/ pH unit is observed. These results are consistent with a number of previously reported results[1] (37, 19, 41-43).

The observed midpoint reduction potential can be pH

[1]*The data in reference 41 were recalculated using a value of n = 1 in the Nernst equation. The data in reference 42 were recalculated using 0.435 as the ratio of extinction coefficients of reduced to oxidized ferredoxin at 425 nm.*

Table II. $E_{1/2}$ vs. pH data for *Clostridium pasteurianum* ferredoxin

$E_{1/2\ (obs)}$ are experimentally determined values.

$E_{1/2\ (fit)}$ are values generated by FITFUNCTION (see text)

pH	$E^{*}_{\frac{1}{2}}$ *(obs)*	$E_{\frac{1}{2}}$ *(fit)*
6.53	-374.5mv	-374.0
6.82	-378.8	-376.7
7.082	-380.3	-380.5
7.41	-387.1	-388.0
7.773	-400.2	-400.0
8.16	-417.7	-415.4
8.41	-422.2	-425.5
8.751	-438.9	-437.7

*estimated uncertainties $_{(average)}$ = 1 mv.

dependent if the protein conformation itself is pH dependent or if the oxidation-reduction equilibrium involves hydrogen ion binding. The change in conformation must, of course, be coupled to some alteration of the redox active center (the 4 Fe-4S centers), whether this coupling changes the bonding geometry at that site or changes the electrostatic environment of that site. In order to test for a pH dependent protein conformation, nmr, epr and CD spectra were recorded as a function of pH.

The ^{1}H-nmr spectra of oxidized *C. pasteurianum* ferredoxin at pD 6.8 and pD 8.9 appear in Figure IX. The resonances in the 0-8 ppm region (from DSS) arise from aromatic and aliphatic side chains in the polypeptide and because of poor resolution, provide no clear insight into the cluster geometry. The relevant resonances appear in the 10-20 ppm region of the spectra. These resonances arise from the β-cysteinyl protons which appear as single proton resonances shifted downfield due to the paramagnetism of oxidized 4Fe-4S centers at room temperature (21). The position of these resonances depends on contact shift interaction and are thus sensitive to the orientation of the β-carbon to sulfur bonds in the cysteinyl ligands at the clusters (44). The resonance positions exhibited by the β-cysteinyl protons therefore, provide information concerning the geometry of Fe-S centers and their neighboring atoms. Thus, the ^{1}H-nmr spectra are useful in

identifying conformation changes at the cluster. As can be seen from Figure IX, the resonances in the 10-20 ppm region occur at identical field strengths at pD 6.8 and pD 8.9. It therefore can be concluded that no detectable conformation differences exist at the immediate environment of the clusters in oxidized *C. pasteurianum* ferredoxin between these two pD values.

To further examine conformation as a function of pH and to include the reduced state of ferredoxin, epr spectra were compared at the pH values shown in Figures X and XI. Two sets of spectra appear; the first pair (Figure X) are the spectra of partially reduced ferredoxin samples (approximately 15% reduction); the second pair (Figure XI) are the spectra of more fully reduced ferredoxin samples (approximately 75-90% reduced). Slightly reduced samples of ferredoxin exhibit spectra which arise predominantly from reduced 4Fe-4S centers in one-electron reduced molecules. Fully reduced samples exhibit a more complex spectrum which arises from interaction between two paramagnetic reduced 4Fe-4S centers in the same molecule (20). In either case, the linewidths and g-values of the signals are sensitive to the bonding geometry of the 4Fe-4S centers. The spectrum of slightly reduced samples in Figure X exhibits a small component of the two-electron reduced spectrum, visible as small peaks centered around 3.3 KG. These features correspond to the two major peaks of

Figure IX. The 270 MHz ^{1}H-nmr spectra of oxidized *Clostridium pasteurianum* ferredoxin

Conditions: temperature, 22°; buffer, 0.04 M potassium phosphate-d, in D_2O, pD as labeled

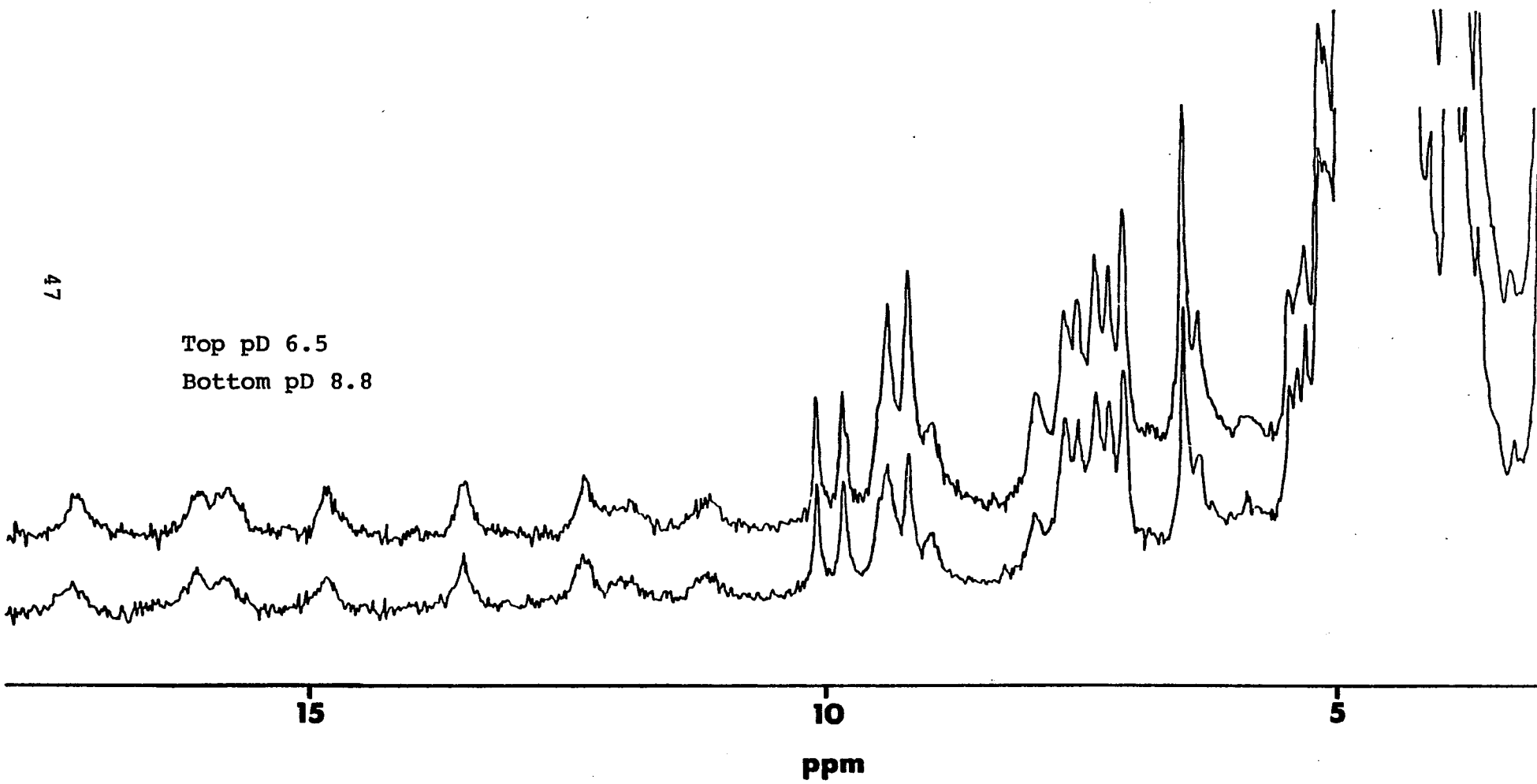

Top pD 6.5
Bottom pD 8.8
15
10
5
ppm

Figure X. Epr spectra of partially reduced samples of *Clostridium pasteurianum* ferredoxin at pH 7.01 and pH 8.99

Conditions: temperature, 13 K; gain, 500 (pH 7 spectrum), 800 (pH 9 spectrum); modulation amplitude, 2 G; power, 5 mW; ν, 9.402 GHz; ferredoxin concentration, ≈ 0.5 mM; buffer, 0.04 M potassium phosphate in 0.5 M NaCl

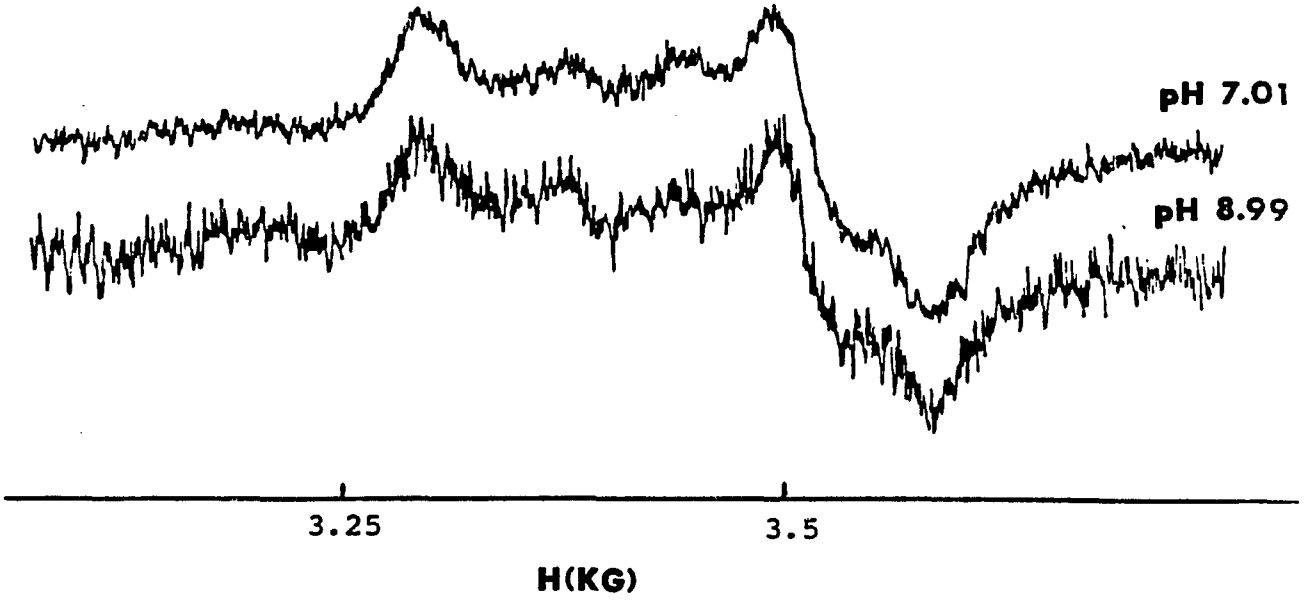
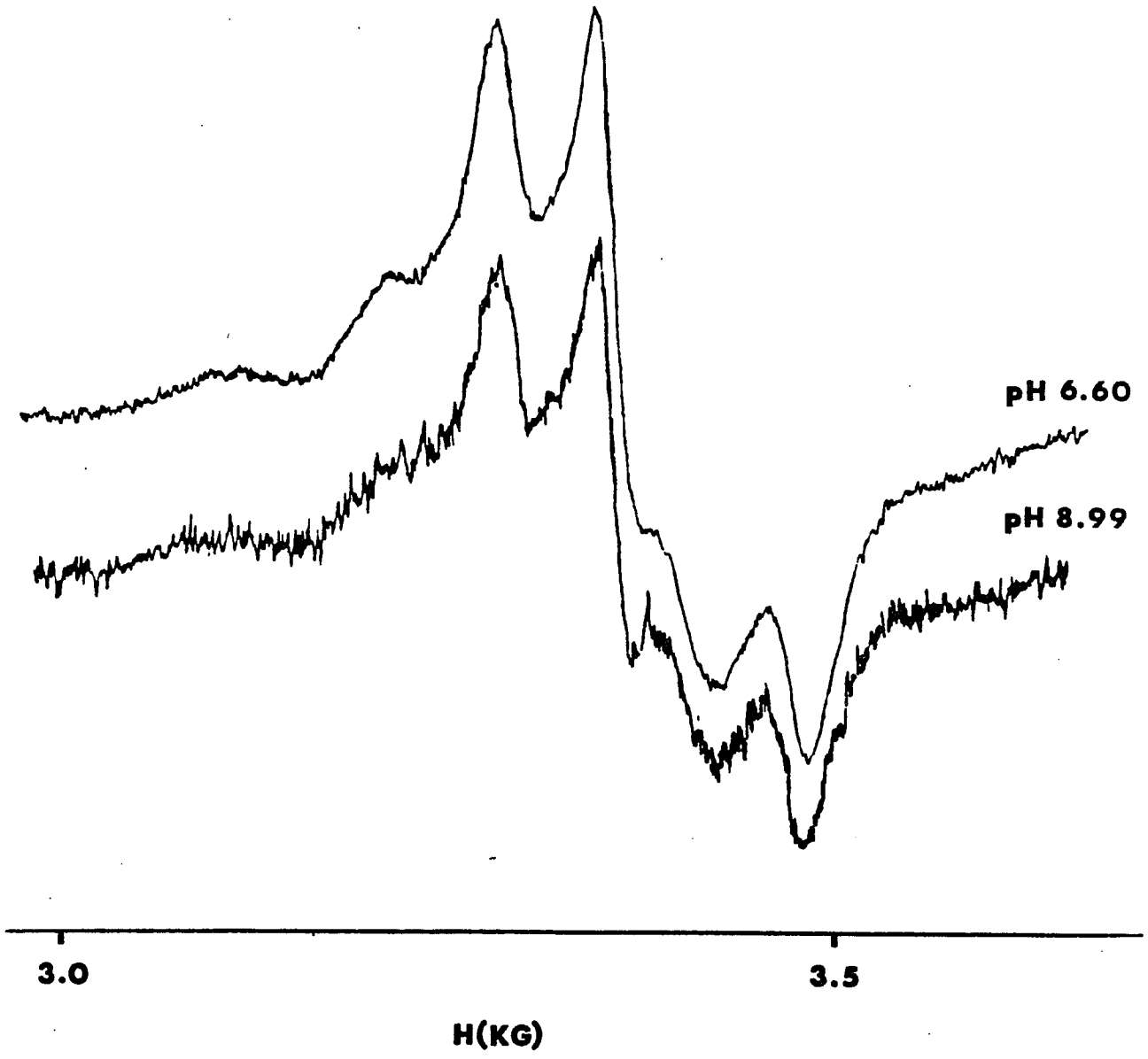

pH 7.01
pH 8.99
3.25
3.5
H(KG)

Figure XI. Epr spectra of near fully reduced samples of *Clostridium pasteurianum* ferredoxin

Conditions: same as figure X except gain, 320, for both spectra


pH 6.60
pH 8.99
3.0
3.5
H(KG)

the fully reduced ferredoxin spectrum.

The observation that the spectra in Figures X and XI are essentially superimposable at the pH values given, indicates that not only is the geometry at a single cluster conserved (partially reduced samples) but also that the conformations at pH 7 and pH 9 are similar enough to conserve the spin interaction between paramagnetic centers in the same molecule. (The small differences between the spectra of the two near-fully reduced ferredoxin samples may be ascribed to small differences in the percent reduction. Though the samples were identically prepared, it is difficult to exactly reproduce an equal percentage of reduced species in two different samples).

The epr data corroborate the ^{1}H-nmr findings, indicating that the conformation of *C. pasteurianum* ferredoxin is pH independent. CD spectra of oxidized ferredoxin were recorded at pH 6.5 and pH 8.5 and were found to be identical. The CD spectrum of reduced ferredoxin from 300 nm to 800 nm was also pH independent from pH 6.5 to pH 8.3 (see Figures XII and XIII).

The spectroscopic results eliminate the possibility that a pH dependent conformation equilibrium is the origin of the observed pH dependent $E_{\frac{1}{2}}$ and leave the alternative, that it is hydrogen ion binding which gives rise to the effect. A pH dependence can be incorporated into the Nernst

Figure XII. The CD spectra of oxidized *Clostridium pasteurianum* ferredoxin

A: pH, 8.5

B: pH, 6.5

Buffer: 0.1 M Tris-HCl/ 0.5 M NaCl

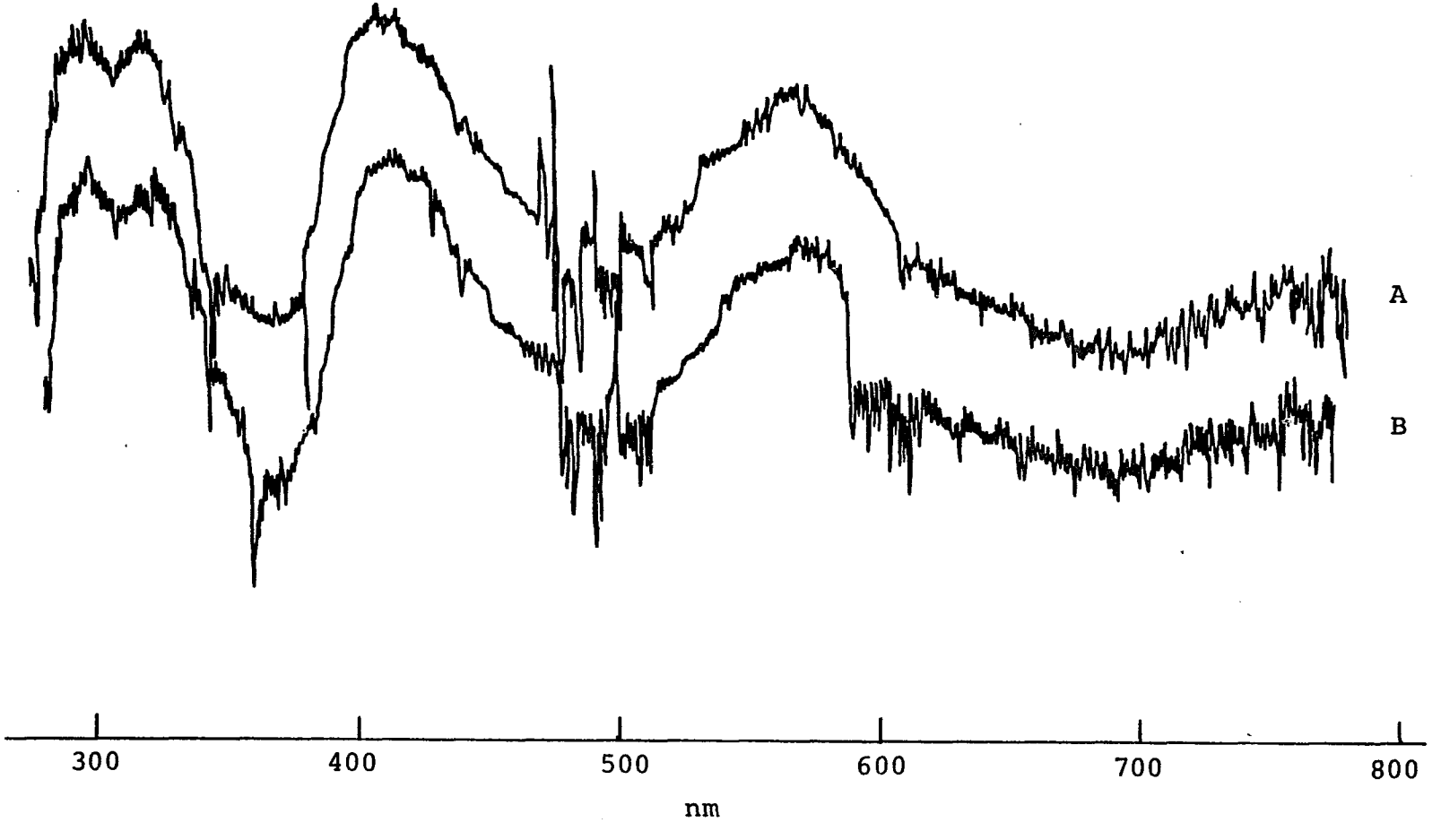

A
B
300
400
500
600
700
800
nm

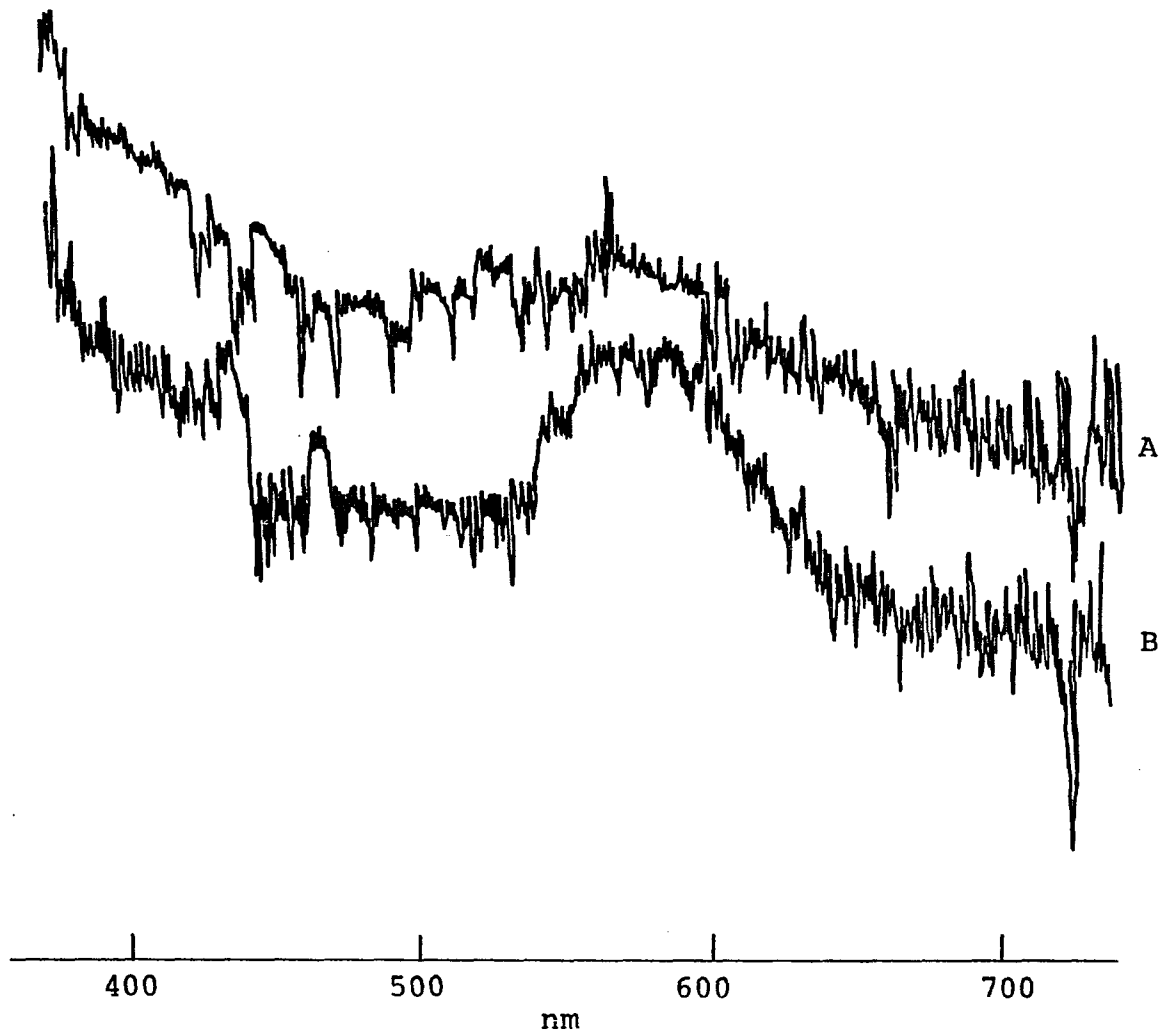
Figure XIII. The CD spectra of reduced *Clostridium pasteurianum* ferredoxin

A: pH, 6.5

B: pH, 8.3

Buffer: 0.1 M Tris-HCl/ 0.5 M NaCl

Samples were reduced with a 20 to 30-fold excess of sodium dithionite in the same buffer.


A
B
400
500
600
700
nm

equation for reduction of ferredoxin using the following model (45):

$$(4Fe\text{-}4S)_{ox} + e^- + H^+ \overset{E^a_{1/2}}{\rightleftharpoons} (4Fe\text{-}4S)_{rd} + H^+$$

$$\uparrow\downarrow K_{ox} \qquad\qquad \uparrow\downarrow K_{rd}$$

$$(4Fe\text{-}4S)_{ox}\text{—}H^+ + e^- \overset{E^b_{1/2}}{\rightleftharpoons} (4Fe\text{-}4S)_{rd}\text{—}H^+$$

The diagram above is only schematic. It is not required in such a scheme that the site of hydrogen ion binding be the 4 Fe-4S center, but only that the equilibrium for binding be oxidation state dependent. $E^a_{1/2}$ and $E^b_{1/2}$ are the respective midpoint reduction potentials of the unprotonated and protonated forms. K_{ox} and K_{rd} are the equilibria for proton association to the oxidized and reduced forms of the protein. For a one electron reduction at 25°, the apparent midpoint reduction potential in millivolts is given by the following:

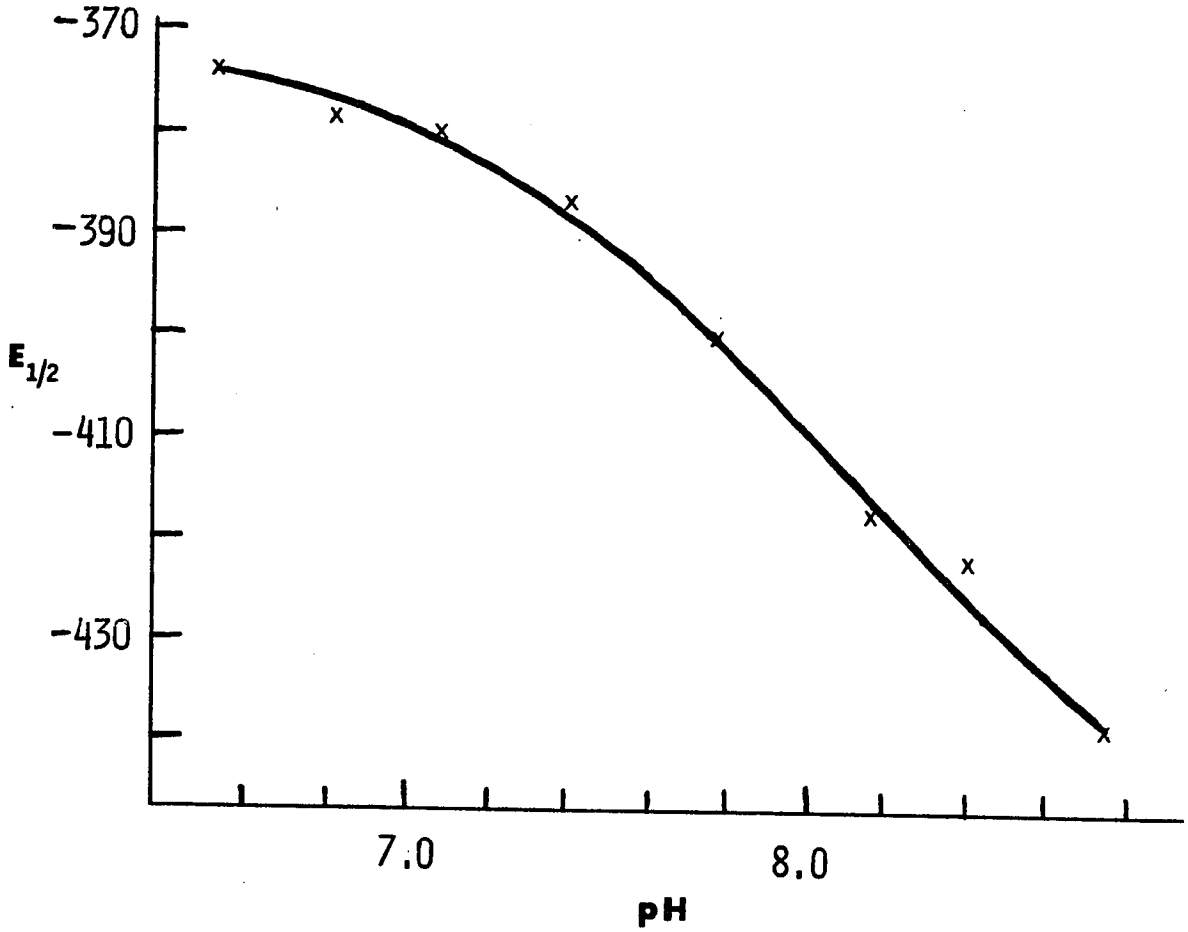
$$E_{1/2} = E^b_{1/2} - 59 log \frac{K_{rd}(1 + K_{ox}(H^+))}{K_{ox}(1 + K_{rd}(H^+))} . \qquad \text{(EQ 1)}$$

The equation is derived based on the definitions of the equilibrium constants above (proton association equilibria) and the conditions of equilibrium for the reduction (amount of reduced species is equal to the amount of oxidized species).

Figure XIV is a plot of the experimental data along with a superimposed curve which was calculated using equation 1.

Figure XIV. $E_{\frac{1}{2}(apparent)}$ vs. pH for *Clostridium pasteurianum* ferredoxin.

All solutions contained 0.03 mM ferredoxin, 0.1 M Tris-Acetate-Glycine-Phosphate, 0.5 M NaCl. x = experimentally determined $E_{\frac{1}{2}}$ values. Solid line is a curve calculated from Equation 1 with the parameters given in the text.


-370
-390
-410
-430
$E_{1/2}$
7.0
8.0
pH

An excellent fit is obtained using a model describing two equivalent sites of protonation per molecule of ferredoxin, each with a $pK_{ox} = 7.4 \pm 0.1$ and $pK_{rd} = 8.9 \pm 0.2$. (The possibility of more than two sites of protonation cannot be excluded, however, and the additional variable parameters would certainly give as good a fit to the experimental data as the simple case does). The data were used as input to the FITFUNCTION statistics package in the PROPHET system. The system performs multiple iterations of curve fitting trials using the equation supplied to FITFUNCTION and also generates standard deviations from which the limits on the pK values were calculated.

Based on the pH independence of the protein conformation and the quality of the fit of the experimental data in Table II to the calculated curve, it is concluded that the origin of the pH dependence of the midpoint reduction potential is oxidation state dependent hydrogen ion association.

The oxidation state dependent pK values obtained from the $E_{\frac{1}{2}}$ vs. pH data may be assigned to sites on the iron-sulfur centers, namely the cysteinyl or acid-labile sulfur atoms. The 4Fe-4S cluster hydrogen ion equilibrium would be intrinsically oxidation state dependent because reduction of the center would increase the basicity of a sulfur atom and thereby shift the pK to higher values (7.4 to 8.9). The following observations are consistent with or directly suggest

hydrogen ion association to iron-sulfur centers:

1. With the exception of *Chromatium* HiPIP, all the reported values for the pH dependence of iron-sulfur protein reduction potentials are negative, despite a wide variation in peptide composition. This fact suggests that the pH dependence is a property of the iron-sulfur center, not the polypeptide chain.

2. *Chromatium* HiPIP does not exhibit a pH dependent reduction potential in the range from pH 7 to pH 11 (38). It has been shown by Peisach, et al. (46), that the 4Fe-4S center in this protein is inaccessible to solvent at pH 7 and is therefore unable to participate in a protonation equilibrium with water.

3. A pK_a of 7.4 has been reported for the water soluble synthetic analogue $Fe_4S_4(SCH_2CH_2COO)_4^{6-}$ (48).

(It should be noted that evidence for cluster protonation does not appear in the nmr or epr spectra at low pH (less than 7) but consideration of the nature of these spectra indicates that the effects of protonation may not be observed. For example, if the site of protonation is a cysteinyl sulfur atom, the nmr resonance positions of the β-cysteinyl protons associated with that residue would be expected to change. However, in the nmr spectrum of the oxidized ferredoxin, only eight of the sixteen β-cysteinyl proton resonances are resolved and therefore, protonation of a cysteinyl sulfur atom

would not necessarily be detected. In the epr spectra, hyperfine interaction would be present between a proton bound to a cysteinyl sulfur atom and the unparied spin in the cluster but it is likely that this interaction is too small to be observed in a frozen solution spectrum with significant g-value anisotropy. If the site of protonation were an acid-labile sulfur atom, it is unlikely that any effect on the β-cysteinyl proton resonances would be observed in the nmr spectra. In the epr spectra for this second case, a larger hyperfine interaction might be expected but it is still reasonable that the effect is too small to be detected).

Although titrable sites other than the cysteinyl sulfur atoms are present on the polypeptide chain, none could exhibit the oxidation state dependent pK's found from Figure XIV. The titrable sites are aspartic acid residues 6, 27, 33, 35, and 39; glutamic acid residues 17 and 55; residue 3 which is lysine; and the carboxyl and amine terminals (13). Of these, only Lys and the N terminal could have a pK near 8.9 and though a pK of 7.4 is also reasonable for the N terminal, it is not probable for the lysine. For example, an intrinsic pK as low as 8.4 has been assigned to Lys^{42} in sperm whale ferrimyoglobin because it is hydrogen bonded to a backbone carbonyl and has limited solvent accessibility (48). Even if such special circumstances ex-

isted for the lysine in *C. pasteurianum* ferredoxin, a pK of 7.4 remains unlikely.

Mechanisms through which the pK of the lysine or N terminal could be varied may be proposed, but none is likely to be in operation in *C. pasteurianum* ferredoxin. For example, electrostatic effects certainly could alter pK values and the shift would be toward more basic values upon reduction. However, the overall charge on the ferredoxin between pH 6 and pH 9 is negative due to the preponderance of acidic residues and the increase in negative charge on reduction could only have a small influence on the pK. Furthermore, the estimated distances between the lysine residue or the N terminal and the center of the nearest Fe-S cluster (10.2 and 8.7 $\mathring{A}$ respectively, estimated from the x-ray structure of *P. aerogenes* ferredoxin (49)) would seem to preclude any electrostatic influence provided by the increase in cluster charge. Calculations of the change in the pK of the NH_2 terminal (valine) in sperm whale ferrimyoglobin with large changes in pH (i.e., variation of the pK with large changes in overall protein charge from pH 4.5 to pH 9.5) yield a maximum of only a 0.4 unit pK shift (50).

There may also be a special mechanism to alter the pK of the N terminal upon reduction in this ferredoxin. The NH_2 terminal (alanine) in *P. aerogenes* ferredoxin partici-

pates in an ion pair with the carboxyl terminal and it is likely that such an ion pair also exists in *C. pasteurianum* ferredoxin, considering the extensive sequence homologies and similarities in their spectral properties. The electrostatic influences of an ion pair involving the N terminal could conceivably be part of a mechanism to alter the pK through a conformation change on reduction, but several implications of such a mechanism are inconsistent with known properties of this protein. If the clusters are indistinguishable in this hypothetical conformational coupling mechanism, it is implied that the midpoint reduction potentials for the two clusters must exhibit cooperativity. It is known, however, from epr studies, that the cluster potentials in *C. pasteurianum* ferredoxin are not cooperative (51). It can also be proposed that reduction of a specific one of the two clusters is required for the conformational coupling, but it is then not possible to simulate the experimental pH dependence of $E_{\frac{1}{2}}$ using equation 1. Note that in this second case, the $E_{\frac{1}{2}}$ of the two clusters would differ because of the effect of the ion pair on one cluster in particular. At high pH values, the N terminal would be uncharged and therefore the cluster potentials should be the same. It is for this reason (equivalence of cluster potentials at high pH) that it is not possible to fit the experimental data. Also inconsistent

with this model is the fact that the cluster potentials are known to be nearly identical at low pH. ($E_{\frac{1}{2}}$'s for reduction of clusters in the same molecule are within 10 mv of each other (52)).

The evidence presented here, in conjunction with the generally observed negative dependence of $E_{\frac{1}{2}}$ on pH, indicates that binding of hydrogen ions to iron-sulfur centers is likely to be a general feature of the chemistry of iron-sulfur proteins. Indeed, the enzymatic activity of hydrogenase, and the involvement of iron-sulfur protein centers in energy conservation at Site I in the mitochondrial electron transport chain may represent specializations of this aspect of iron-sulfur protein chemistry.

D. Additional experiments

1. Attempt to form a carbon monoxide adduct of *Clostridium pasteurianum* ferredoxin

The pH dependence of the midpoint reduction potential in *C. pasteurianum* ferredoxin has been examined and these results appear in section III. There is no direct evidence however, contained in the description of experiments related to the pH dependence, for protonation of 4Fe-4S centers, though all the evidence accumulated is consistent with that conclusion. An approach was taken to demonstrate indirectly that the acid-base properties of the 4Fe-4S centers do in fact give rise to the observed pH effect based on properties reported for the iron-sulfur enzyme hydrogenase.

Hydrogenases are known to bind carbon monoxide which is a competitive inhibitor of enzyme activity (16, 53, 54). For some enzymes, the binding is light reversible (55, 56). There is substantial evidence that the binding of CO occurs at the iron in the 4Fe-4S center(s) in these enzymes. Foremost is the evidence that a significant change in the epr spectrum of both oxidized and reduced *C. pasteurianum* hydrogenase occurs after treatment with CO (54). Additionally, there is a 2.4 G broadening of the line with g-value = 2.009 in oxidized hydrogenase treated with ^{13}CO and a 2 G broadening for the g = 1.93 signal of reduced hydrogenase in the presence of ^{13}CO (54). The line broad-

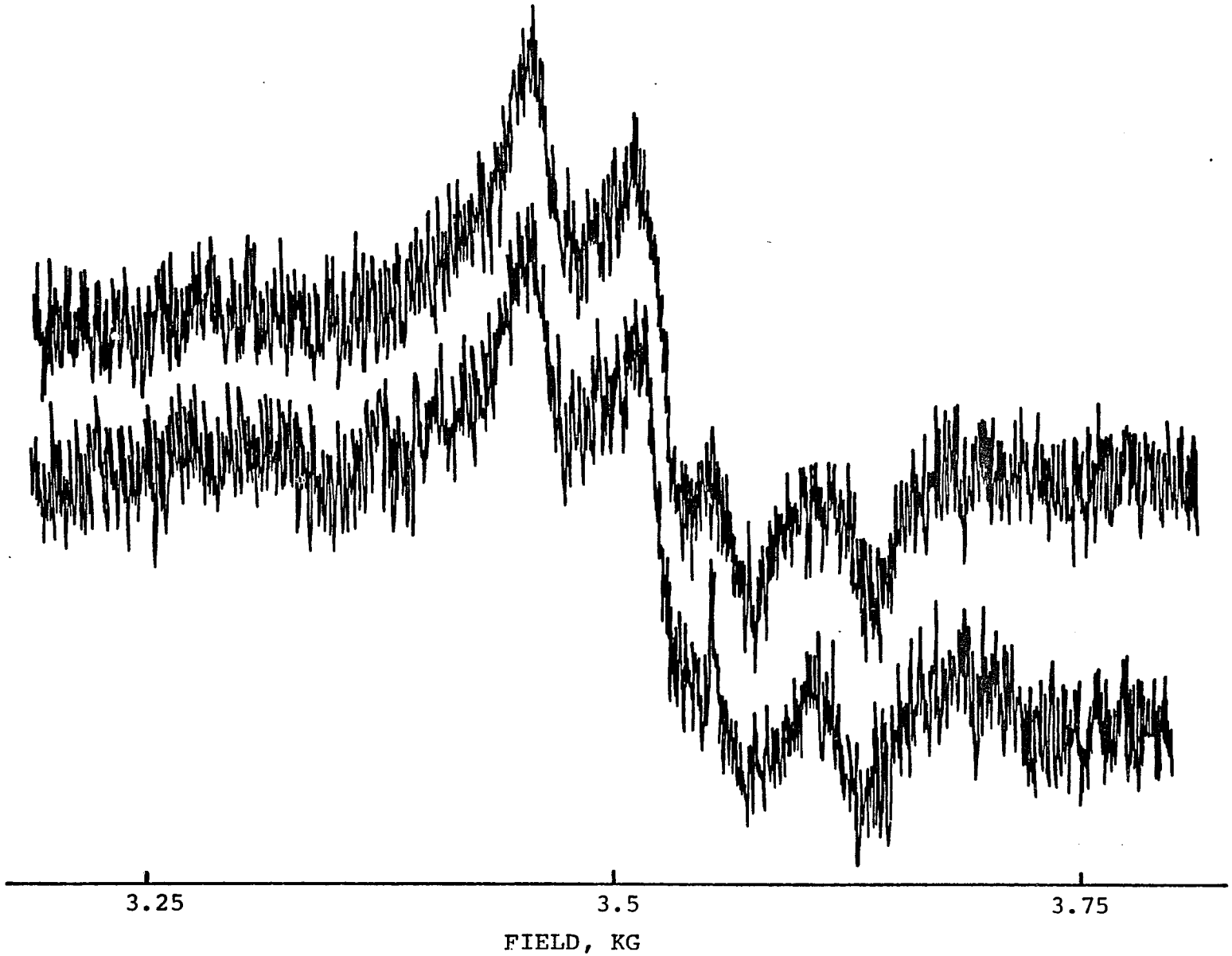
ening results from unresolved hyperfine interaction between the unpaired spin and the spin of the ^{13}C nucleus ($I = \frac{1}{2}$). Also consistent with iron-carbonyl formation in the enzyme adduct is the fact that iron-carbonyls may be photo-dissociated (57) just as the enzyme inhibition may be light-reversed.

The binding of CO to the iron-sulfur centers in hydrogenase suggested a means to probe the pH dependence of *C. pasteurianum* ferredoxin reduction potentials. The pH dependence of a ferredoxin-CO adduct would be expected to differ from the pH dependence exhibited by the native protein. The introduction of a carbonyl ligand in the cluster (which should then exhibit a new pH dependence) would provide a route to the demonstration of Fe-S center involvement in pH effects. It was found, however, that *C. pasteurianum* does not bind CO. This conclusion is based on the observation that no differences are observed between the epr spectrum of native ferredoxin and ferredoxin which was reduced in the presence of carbon monoxide. (See Figure XV).

The active site in hydrogenase is a 4Fe-4S center (54, 16). Certainly, there are significant differences between the chemistry of 4Fe-4S centers in the enzyme and the 4Fe-4S centers in ferredoxin since the ferredoxins do not exhibit any hydrogenase activity. The evidence that CO

Figure XV. Epr spectrum of *Clostridium pasteurianum* ferredoxin in the presence of carbon monoxide and control spectrum

Conditions: temperature, 15 K; gain, 1250; modulation amplitude, 2 G; power, 2 mW; ν, 9.3795 GHz; buffer, 0.1 M Tris-HCl/ 0.1 M NaCl, pH 7.4


3.25
3.5
3.75
FIELD, KG

does not bind to the ferredoxin clusters is immediate evidence of the differences in the nature of the iron binding in these two proteins. Some speculation concerning the details of the hydrogenase mechanism with the evidence of the CO binding in mind, along with the already known facts about the hydrogenase mechanism, is contained below.

Krasna (16) has shown that the reduced, active form of hydrogenase is an enzyme hydride. He has further suggested that the mechanism of hydride formation would be facilitated by some basic group near to the cluster capable of binding the proton released in the heterolytic cleavage of H_2. It is suggested that the iron in hydrogenase clusters is more labile than that in ferredoxin clusters and this lability is directly related to the enzyme mechanism. (Note also that hydrogenases are very oxygen sensitive whereas ferredoxins have a much higher stability in the presence of oxygen). The reversible binding of CO suggests that either ligand exchange can occur at the iron atoms in clusters or that the iron can become penta-coordinate in hydrogenase clusters. The stoichiometry and kinetics of binding are not known and therefore, both of these possibilities must be considered. Also, the reversibility indicates that the Fe-S cluster must remain essentially intact, suggesting that the acid-labile sulfurs are not dissociated by CO binding.

A mechanism for the formation of an enzyme hydride with H_2 as the substrate can be described in the following way: the dissociation of a cysteinyl sulfur ligand upon H_2 binding would provide a strongly basic site for protonation and hydride formation is thus facilitated. Such a hypothetical mechanism might be favored over one in which the hydride occupies a fifth ligand position on an iron because the cysteinyl sulfur would not be as good a base as it is when dissociated. A pH dependence of H_2 binding (reduction) exhibited near the pK of cysteinyl sulfur would provide some evidence that such a mechanism operates in hydrogenases. Also, experiments to determine the kinetics and stoichiometry of binding of cysteine-titrating reagents (e.g., mercurials) would also provide insight into the details of the mechanism. Such experiments would determine if dissociation of cysteine does in fact occur.

2. Synthesis of $Fe_4S_4(SCH_2CH_2COO)_4^{6-}$

The synthesis of the 4Fe-4S analogue $Fe_4S_4(SCH_2CH_2COO)_4^{6-}$ was undertaken to provide a soluble model which was expected to exhibit a pH dependent midpoint reduction potential. The demonstration of such a dependence would have provided conclusive evidence that iron-sulfur centers give rise to the dependencies exhibited by the Fe-S proteins. Difficulties

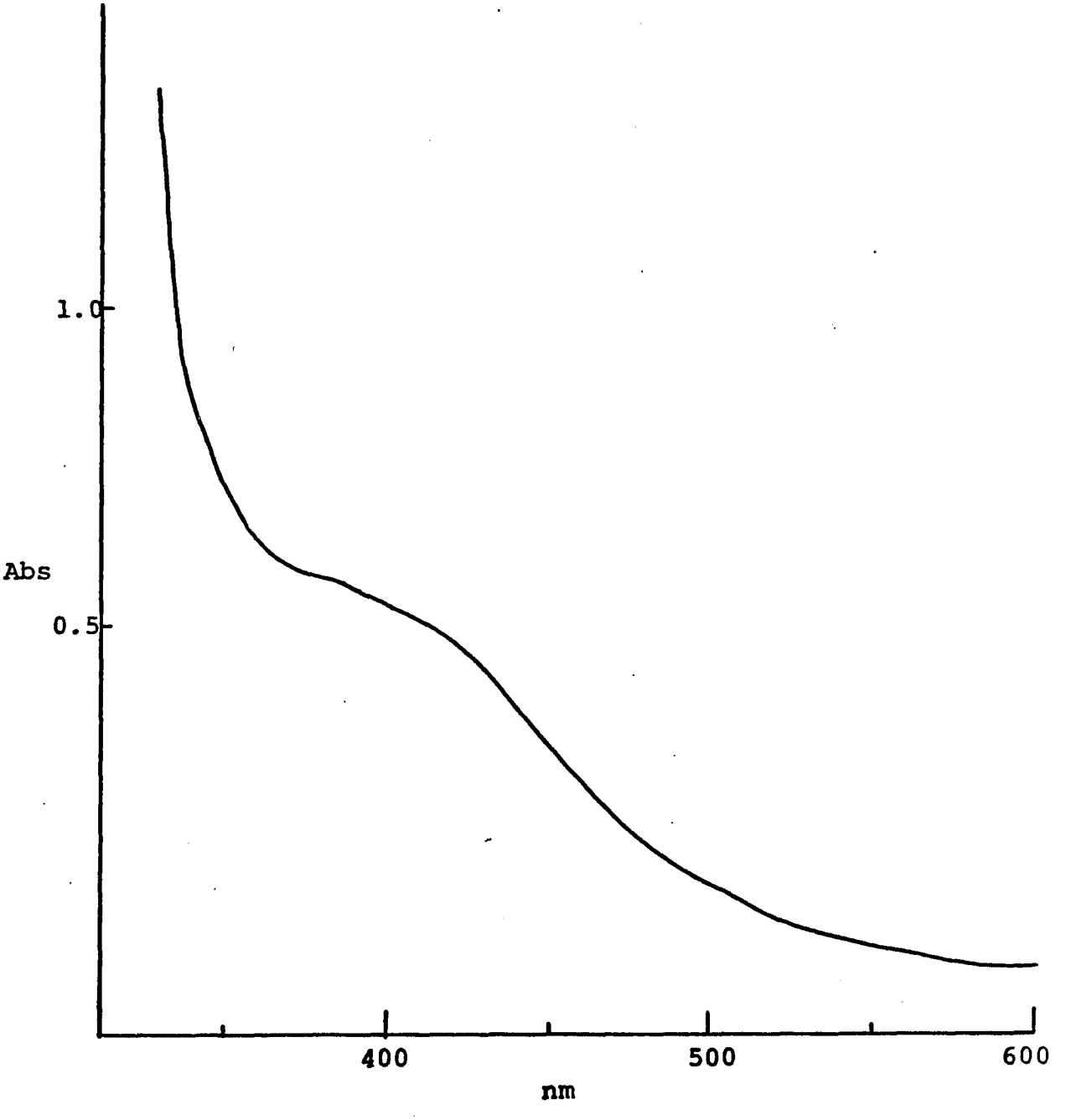
associated with handling of the product, however, precluded completion of the polarographic analyses for the determination of $E_{\frac{1}{2}}$. The model was observed to be extremely air sensitive in solutions in which the concentration of thiolate ligand (β-mercaptopropionic acid) was not very much larger than the concentration of the model. Solutions containing an approximately 100 fold excess of ligand, however, were stable for 2 weeks.

The following procedure was used as a synthetic route to the water soluble model first described by Carrell, et al. (58).

All solutions were de-aired using oxygen free grade nitrogen to bubble through needles into vessels which were sealed with serum caps. The reaction vessel consisted of a 250 ml three neck flask fitted with a condenser, a pressure equalizing dropping funnel, and a serum cap. The condenser outlet led to a one-way bubbler valve. Nitrogen was passed into the vessel through a needle fitted through the serum cap. A solution containing 4.1 g $NaOCH_3$ and 4.0 g β-mercaptopropionic acid in 50 ml methanol was bubbled for 45 minutes. To this de-aired solution was added 33 ml of a de-aired solution of 2.5 g $FeCl_3$ in 50 ml methanol, through the pressure equalizing funnel over a 30 minute period, with stirring. The solution turned purple as $FeCl_3$ solution entered and then turned olive green after all the $FeCl_3$ had

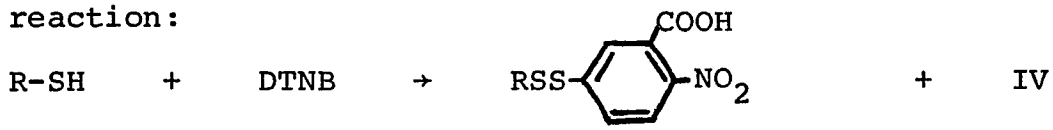
been transferred. A solution of anhydrous sodium sulfide was prepared in the following way: H_2S was bubbled through 1 g of $NaOCH_3$ in 50 ml methanol for approximately 5 minutes. Nitrogen was then bubbled through the same solution for 15 minutes to remove excess H_2S and a second portion of $NaOCH_3$ (1 g) was added. Thirty-five ml of this solution was added by syringe, to the reaction vessel. The reaction mixture at this time, was black with some black precipitate. Then, 2.5 g of tetrabutylammonium bromide in 10 ml methanol was added and the mixture refluxed for 4.5 hours. All the solvent was then removed *in vacuo*. The reaction vessel, which now contained a gray powder, was filled with nitrogen and transferred into the glove box prepared as described in section II, G. Then, 25 ml methanol and 25 ml N-methylpyrrolidine were added to the gray powder and stirred for 10 minutes. The dark brown solution was filtered and a second trituration was performed with 10 ml methanol and 10 ml N-methylpyrrolidine. Then, 50 ml N-methylpyrrolidine was added to the combined filtrates. Very fine, dark brown crystals appeared after approximately 12 hours and the product was filtered (yield ≈ 1 g). The visible spectrum of this material (50 mg dissolved in 3 ml 0.1 M β-mercaptopropionic acid titrated to pH 9.2 with NaOH) appears in Figure XVI. The spectrum of the material prepared according to the above procedure had the same optical spectrum as

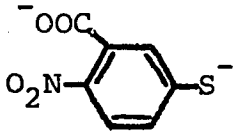
Figure XVI. Optical spectrum of $Fe_4S_4(SCH_2CH_2COO)_4^{6-}$ in 0.1 M β-mercaptopropionic acid/ NaOH, pH 9.2


1.0
Abs
0.5
400
500
600
nm

the material the authors describe (58).

The synthesis was repeated using Schlenck-type glassware for all manipulations. Oxygen-free nitrogen was used as purge gas and was passed through a column packed with Davison Molecular Sieves (Grade 513) and 'GRANUSIC' (granular P_2O_5) to dry the gas. A vacuum manifold was used for purging glassware and all liquids were de-aired by bubbling with nitrogen through a glass sparger. Several purging cycles were performed on the sealed vessels after de-airing and immediately after any transfer. Absolute methanol was fractionally distilled from $Mg(OCH_3)_2$. β-mercaptopropionic acid was fractionally distilled at approximately 3 mm Hg. The distillate was tested with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), Ellman reagent) to determine if significant amounts had oxidized to the disulfide before or during the distillation process. The freshly distilled material gave the calculated absorbance for a stoichiometric conversion of DTNB to the colored anion IV, according to the general reaction:



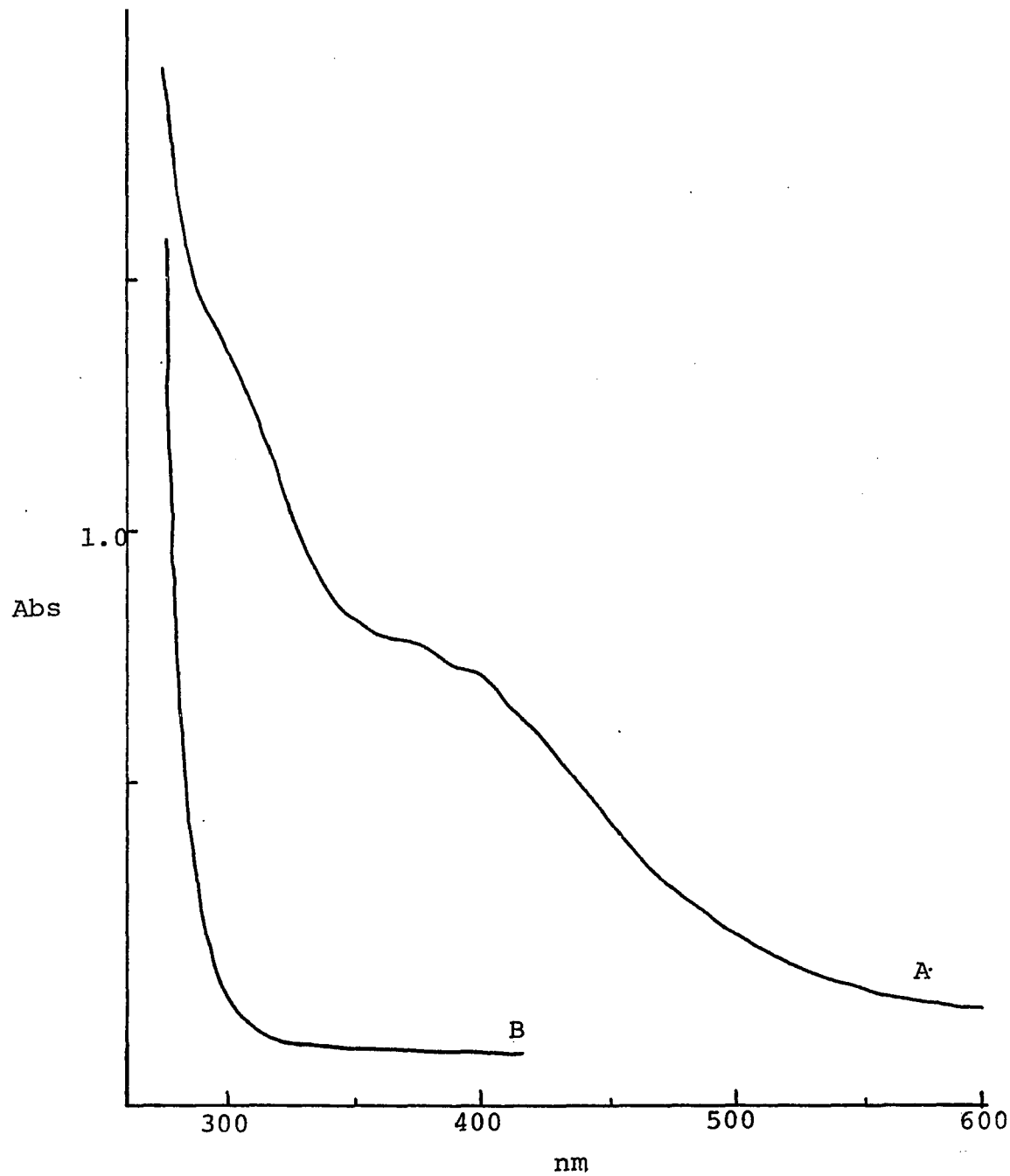


IV

The undistilled material showed only 75% of the stoichiometrically calculated absorbance for conversion of DTNB to the colored product. The $FeCl_3$ solution was filtered through very fine porosity sintered glass before use. The solution of β-mercaptopropionic acid in methanol turned violet as $FeCl_3$ was added and then turned to a deep green slurry as the remainder of the iron was added. The $FeCl_3$ was delivered from a pressure equalizing funnel over a 35 to 40 minute period. After trituration of the gray powder (as in preceding procedure), no crystallization occurred upon addition of N-methylpyrrolidone though the solution was dark brown. The crystallization solution was concentrated in several steps, by evacuation of the vessel to distill away some solvent. Between each evacuation, the solution was left in a methanol-dry ice bath at approximately -30° for several hours or overnight in an attempt to induce crystallization. Finally, a small amount (∿ 10 ml) of acetonitrile was added to the solution and crystallization occurred almost immediately. The product was collected and washed with a small amount of CH_3CN. The optical spectrum of this product appears in Figure XVII, spectrum A. Spectrum B is the optical spectrum of the β-mercaptopropionic acid/ NaOH buffer, pH 9.0. It may be noted that based on the characteristics of the spectra, the material that crystallized overnight (from N-methylpyrrolidine) was pure com-

Figure XVII. Optical spectrum of $Fe_4S_4(SCH_2CH_2COO)_4^{6-}$ in 0.1 M β- mercaptopropionic acid/ NaOH, pH 9.0

Lower spectrum: 0.1 M β-mercaptopropionic acid/ NaOH buffer, pH 9.0


1.0
Abs
A
B
300
400
500
600
nm

pared to the material precipitated by the addition of acetonitrile (in N-methylpyrrolidone). Solutions of the 4Fe-4S compound were stable for approximately two weeks if prepared under thoroughly anaerobic conditions, in the presence of a large excess of buffered β-mercaptopropionate (pH 9).

Discussion

The procedure for the synthesis of $Fe_4S_4(SCH_2CH_2COO)_4^{6-}$ as published, is difficult to reproduce without attention to certain details as discussed here. All solvents must be freshly distilled and water free. β-mercaptopropionic acid may need to be distilled, *in vacuo*, to insure that the reagent is free sulfhydryl. A simple test may be performed using DTNB, which is converted to a colored product with the mercaptan. The extinction coefficient at 412 nm for product, structure IV, is 13,600 $M^{-1} \cdot cm^{-1}$ (59). Any water contained in the purge gas should be removed by passing the gas through dessicants. P_2O_5 is very effective and is conveniently supplied in a granular form (GRANUSIC) which can be packed in a column. Sodium methoxide must be free-flowing and there should be little or no residue when it is dissolved in methanol. $FeCl_3$ solutions must be filtered (through very fine porosity sintered glass) before addition to the reaction mixture (B. Job, personal communication).

The published procedure states that a deep green slurry was obtained upon addition of all the $FeCl_3$. This color was not observed in the synthesis described first, though the product exhibits the same optical spectrum as the product obtained from the second synthesis in which the deep green color was observed. The final crystallization from methanol/N-methylpyrrolidone/acetonitrile gave a powder which was extremely sensitive to air. No crystallization occurred from methanol/N-methylpyrrolidone mixtures even after concentration of solutions by evacuation and chilling to approximately -30°. Storage of solutions containing product over several weeks may result in precipitation of the product in large needles, as described by the authors (58), but this was not observed after a two week period. Addition of acetonitrile (B. Job, personal communication) to the final solution yields immediate precipitation of the product though this material seems impure as evidenced by the small features in the optical spectrum at approximately 400 nm and 375 nm which are absent from other spectra. It may also be noted that the product recrystallized from N-methylpyrrolidine did not exhibit the spectral features which indicate the presence of impurities.

IV. Preliminary Studies of Reconstitution of Iron-Sulfur Centers

A. *Azotobacter vinelandii* Ferredoxin I

There has been no report in the literature of the reconstitution of Fd I from *A. vinelandii*. A procedure has been developed by Hong and Rabinowitz (22) for the 'all or none' reconstitution of clostridial ferredoxins which does not give any iron-deficient products. The following procedure was used to first prepare apoferredoxin from native ferredoxin I:

1. 10 mg Fd I was centrifuged from 90% saturated ammonium sulfate and the pellet was brought up in 15 ml 5% trichloroacetic acid. The suspension was incubated at 0^{o} for one hour.

2. The suspension was spun at 27000 x g for 80 minutes to give a pellet that retained brown color.

3. The brown pellet was resuspended in 15 ml fresh 5% trichloroacetic acid and incubated for 30 minutes at room temperature.

4. The suspension was centrifuged again.

5. The pellet (pale brown) was dissolved in a small amount of 5% sodium carbonate and 4 ml 0.01 M sodium acetate to give a pale brown solution.

6. The solution was dialyzed against 4 liters 0.01 M sodium acetate overnight.

7. The dialysis solution was then changed to 4 liters 0.01 M Na_4EDTA. After 10 hours, the solution was changed to 4 liters distilled water and left overnight. The protein solution retained some brown color and a third precipitation in trichloroacetic acid was performed. The pellet from this precipitation was colorless. It was dissolved in 5% $NaHCO_3$ and dialyzed against water for 2 hours and then 0.25 M Tris-HCl (pH 8.3) overnight.

The reconstitution procedure follows below. All manipulations were performed under Ar.

1. The dialysate from 7 above was made 8 M in urea.

2. 0.210 ml 0.1 M $Na_2S \cdot 9H_2O$ was added.

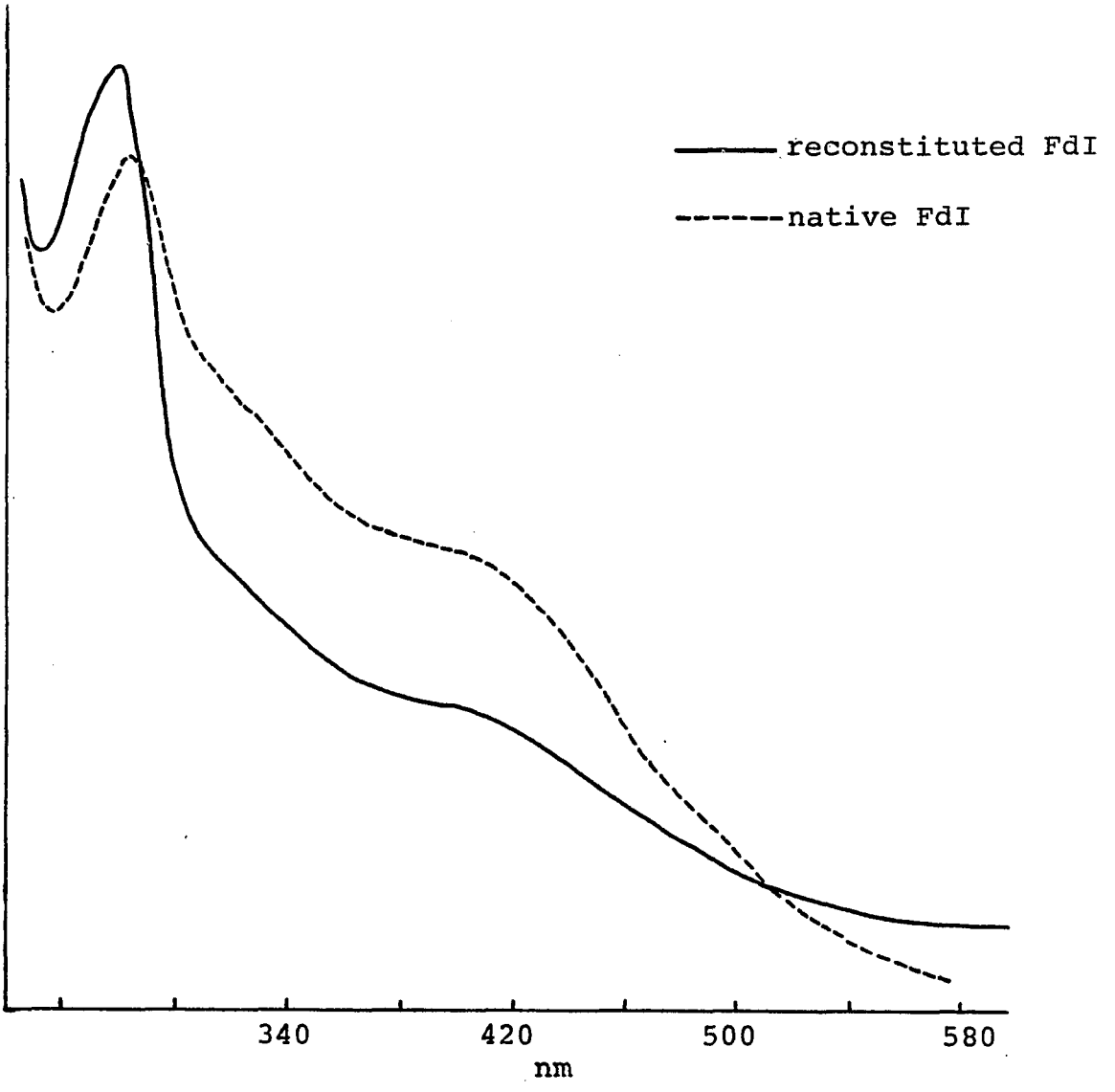
3. 0.18 ml 0.037 M dithiothreitol in 0.1 M Tris-HCl (pH 7.4) was then added.

4. 0.210 ml 0.1 M $Fe(NH_4)_2(SO_4)_2$ was then added.

5. This solution, which contained some black precipitate, was incubated for 30 minutes.

6. The product was diluted aerobically to 60 ml and applied to a DEAE column pre-equilibrated with 0.1 M Tris-HCl (pH 7.4). The band was washed with several column volumes of 0.1 M Tris-HCl (7.4), then with 0.1 M Tris-HCl in 0.25 M NaCl. The protein was eluted with 0.1 M Tris-HCl in 0.5 M NaCl (pH 7.4).

Figure XVIII. The optical spectrum of reconstituted *Azotobacter vinelandii* ferredoxin I


reconstituted FdI
native FdI
340
420
500
580
nm

The optical spectrum of the peak brown fraction eluted from DEAE appears in Figure XVIII. Also in the figure is the spectrum of native ferredoxin I. The purity ratio of Fd I is 0.59 (A_{400}/A_{280}). The ratio exhibited by the reconstituted material is 0.47. The most likely reason for the low ratio here is the presence of unreconstituted apoferredoxin I that co-elutes with the reconstituted protein resulting in a high absorbance at 280 nm. Another possibility is that the reconstituted material was an iron-deficient ferredoxin (which would have an absorbance at 400 nm lower than native protein and thus a smaller purity ratio). The experiments in section V describe attempts to isolate an iron-deficient ferredoxin I.

B. Reconstitution of a synthetic tridecapeptide

There has been some interest in ferredoxins as a class of simple proteins that may have appeared early in the evolution of terrestrial biology. In the interest of demonstrating that a contemporary ferredoxin could have evolved from a simpler, short iron-sulfur protein, a tridecapeptide that mimics part of the sequence of *Peptococcus aerogenes* 2(4Fe-4S) ferredoxin was synthesized by a group at the Space Sciences Laboratory, University of California, Berkeley (Jean Lecocq, Janis Young, Diane Sandlin and Thomas

Figure XIX. The amino acid sequence of three synthetic tridecapeptides and the parallel sequence of *Peptococcus aerogenes* ferredoxin

Peptide I: Ser Cys Ile Ala Cys Gly Ala Cys Lys Pro Glu Cys Pro

Peptide II: Ser Cys Tyr Ala Cys Gly Ala Cys Lys Pro Glu Cys Pro

Peptide III: Ser Cys Ile Tyr Cys Gly Ala Cys Lys Pro Glu Cys Pro

P. aerogenes: Ser Cys Ile Ala Cys Gly Ala Cys Lys Pro Glu Cys Pro (60)

Jukes, unpublished). The two-fold symmetry of clostridial ferredoxins (both in sequence homology from either terminal and in the tertiary structure) suggests that ferredoxins evolved from gene duplication (Jukes, et al., personal communication). This fact suggests that early in evolution, there may have been a short sequence of amino acids that could accommodate a 4Fe-4S center. The three synthetic tridecapeptides from the T. Jukes laboratory have the sequences illustrated in Figure XIX. Also in the figure is the parallel sequence found in *P. aerogenes* ferredoxin. Experiments were undertaken to attempt the reconstitution of an iron-sulfur center in Peptide III, with the following procedure. All steps were performed in a glove box purged with oxygen free nitrogen as described in section II, G. The use of the gas train assembly was not included in the procedure here.

2 mg Peptide III were added to 0.5 ml 0.07 M dithiothreitol and 8 M urea in 0.5 M Tris-HCl, pH 8.3 and the solution was incubated for 2.5 hours. To this was added 0.12 ml 0.1 M $Na_2S \cdot 9H_2O$, then 0.12 ml 0.1 M $Fe(NH_4)_2(SO_4)_2$. Then, 2.36 ml 0.1 M Tris-HCl (pH 7.4) containing 0.035 M dithiothreitol was added and the solution was incubated for 30 minutes. The solution, which contained black precipitate, was diluted to 10 ml with distilled water and was applied to a 4 ml DEAE column prepared in a pipette.

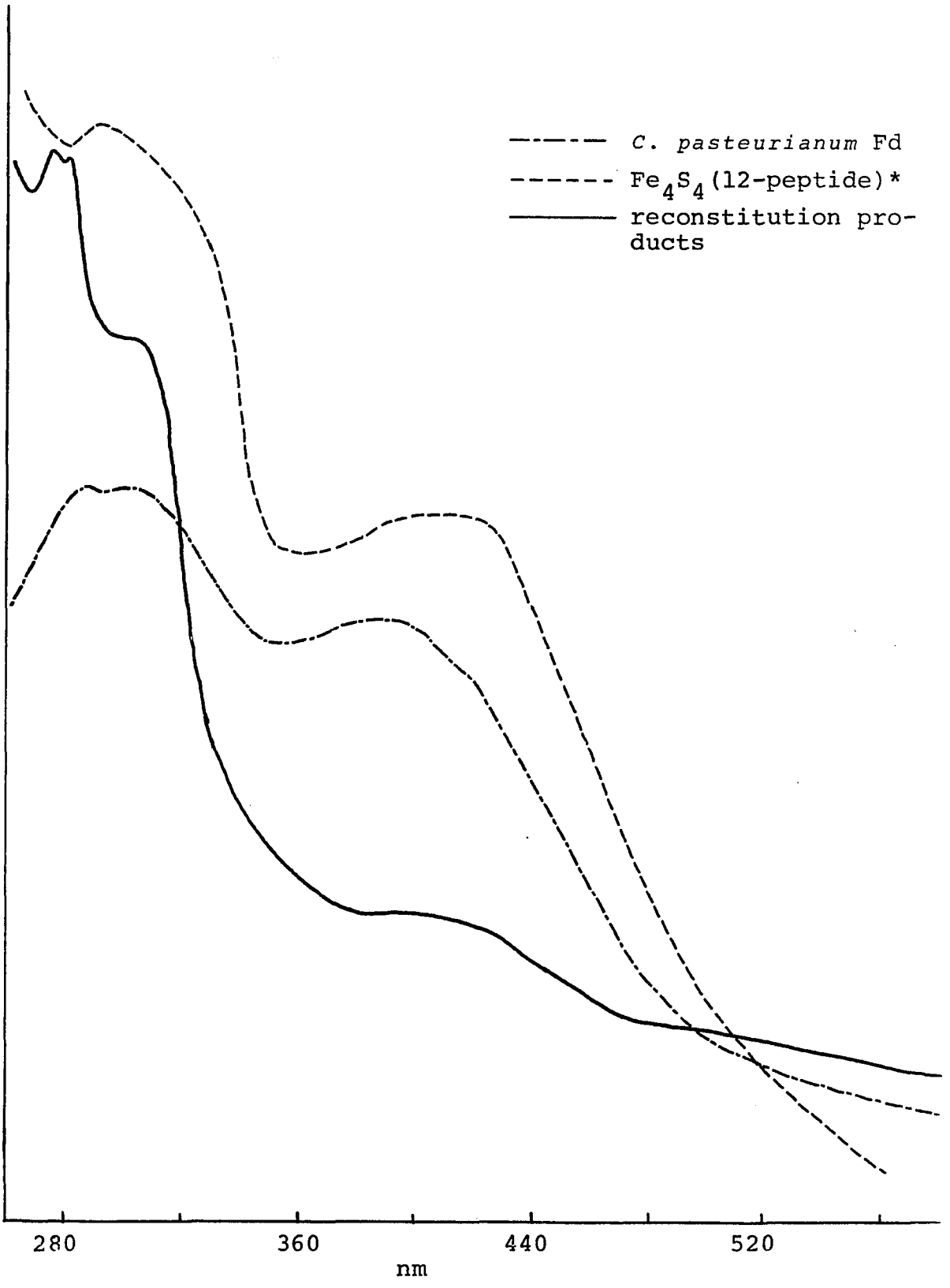
The column had been equilibrated with the following series of solutions (all pH values were 7.4):

1. 10 column volumes 0.1 M Tris-HCl
2. 5 column volumes 0.005 M Tris-HCl containing 0.1 M $Na_2S_2O_4$ (to reduce oxygen in the cellulose)
3. 6 column volumes 0.005 M Tris-HCl in 1 M NaCl
4. 1 column volume distilled water
5. 15 column volumes 0.005 M Tris-HCl

The column was rinsed with a small amount of water after application of the reconstitution products. The product was then eluted in 6 fractions (approximately 1 ml each) with 0.005 M Tris-HCl containing 0.3 M NaCl. The material contained in fraction 2 was placed in a cuvette and sealed with a serum cap. The optical spectrum of fraction 2 appears in Figure XX. A portion of fraction 2 (0.3 ml) was diluted to 1 ml in the presence of air and the spectrum of this solution was recorded over a seven hour period, until the absorbance at 400 nm was near zero. Figure XXI contains the spectrum of this 'aired dilution' after approximately 30 minutes.

The reconstitution procedure was repeated and samples of the colored eluant from DEAE chromatography of the products were prepared as epr samples with varying amounts of reduced methyl viologen and potassium ferricyanide and also without addition of any reductant or oxidant

Figure XX. The optical spectrum of synthetic trideca-peptide III reconstitution product

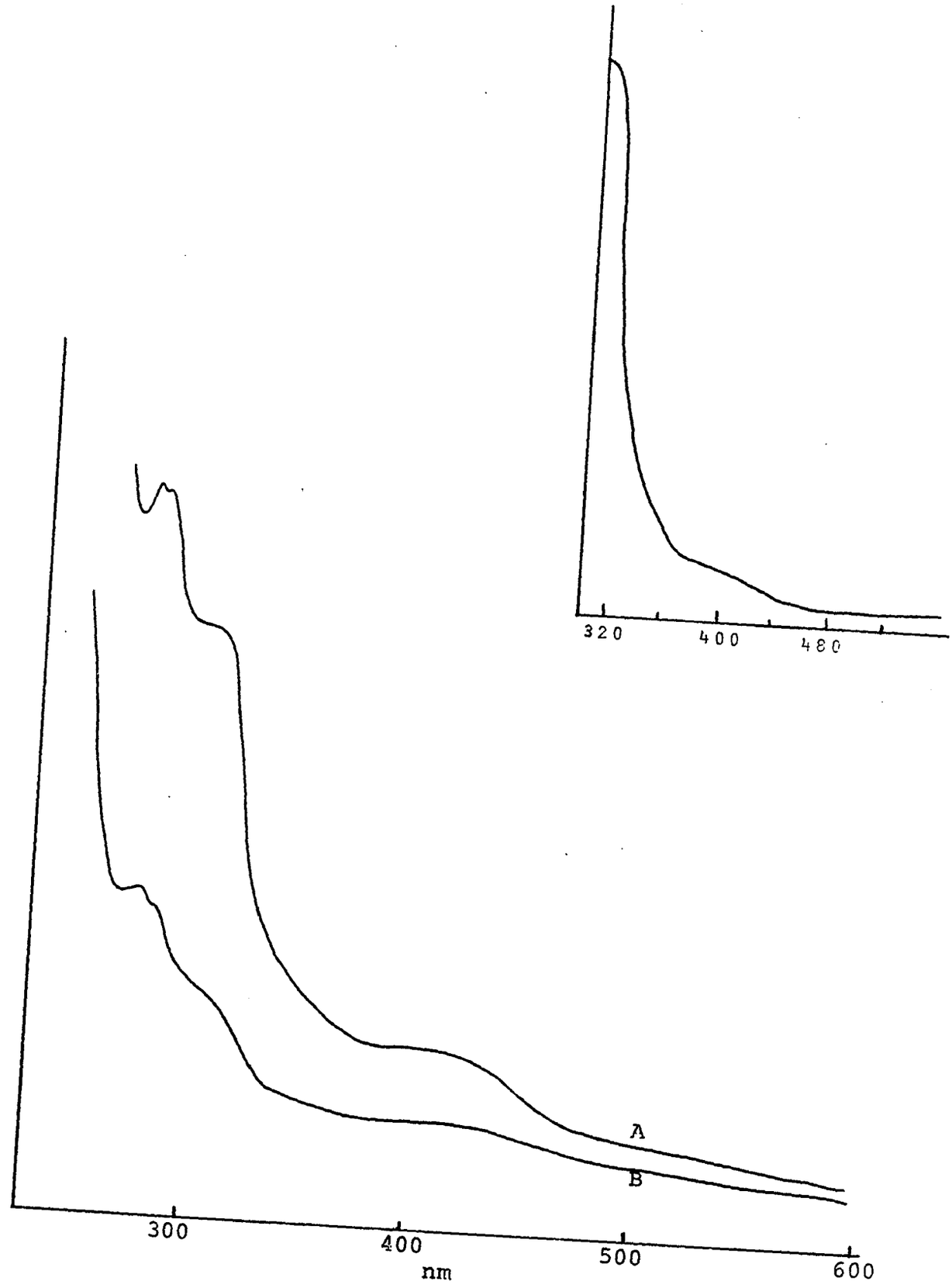


*replotted from (28)

The modified procedures used in these reconstitution experiments are based on the procedure developed by Hong and Rabinowitz. Some related experiments, reported by Que (28) describe the incorporation of a dodecapeptide around a preformed 4Fe-4S center by ligand substitution in dimethylsulfoxide. The reaction involves substitution of t-butyl mercaptide ligands on $Fe_4S_4(S\text{-}t\text{-}butyl)_4^{2-}$ by cysteinyl sulfhydryls on t-BOC-Gly-Cys-Gly-Gly-Cys-Gly-Gly-Cys-Gly-Gly-Cys-Gly-NH_2. Quantitative conversion is reported, afforded by the volatility of the t-butyl mercaptan and DMSO which are removed *in vacuo* to drive the substitution to completion. The spectrum of the Fe_4S_4(12-peptide) appears in Figure XX. Also in the figure are the spectra of *C. pasteurianum* ferredoxin and the reconstitution product of Peptide III. The notable similarities in all three spectra are the broad absorbance bands around 400 nm. Also notable is that the synthetic peptide product exhibits a peak at approximately 313 nm which is absent from the other spectra. This absorbance is exhibited, however, by a solution containing ferrous iron and dithiothreitol as can be seen in the inset of Figure XXI. It seems that the by-product of reconstitution co-elutes with the protein containing fraction from the DEAE chromatography and therefore that the spectrum of the products of reconstitution represent a mixture of an Fe/dithiothreitol complex as well as

Figure XXI. The optical spectrum of synthetic trideca-peptide III reconstitution product after exposure to air

Inset: Optical spectrum of Fe^{2+}/DTT mixture


320
400
480
A
B
300
400
500
600
nm

an Fe-S peptide III complex. Though the spectrum of the products formed simply by mixing Fe^{2+} and dithiothreitol (anaerobically) resembles that of the protein containing fractions from the reconstitution procedure, it is probable that some peptide was reconstituted. The ratio of absorbances at 308 to 400 is 2.84 in the protein containing fraction but is 10.20 in the pure Fe^{2+}/dithiothreitol mixture spectrum. The ratio, A_{275}/A_{400} is 3.5 for the protein containing fraction and 12.5 for the Fe/DTT spectrum. The differences in these ratios corroborate the conclusion that some peptide was reconstituted. An aliquot of the protein containing fraction was diluted aerobically and the spectrum recorded. Immediately upon dilution, the relative absorbance intensity in the range 320-270 nm decreased more than that at 400 (ratio 270/400 → 0.4). Since the Fe^{2+}/DTT complex has strong absorbancy in this range and is very oxygen sensitive, and the absorbance at 400 persists for a short period when the solution is exposed to air, the presence of material with a spectrum characteristic of ferredoxins in the 400 nm region is indicated. Clearly, the spectrum represents a mixture of chromophores and the aliquots prepared for epr did not confirm the presence of an iron-sulfur cluster. The fact that no ferredoxin-like signals were exhibited by these samples does not, however, rule out the presence of reconstituted mater-

ial since the concentrations were very low in the epr samples (estimated to be 10 μM). Additionally, the reconstituted Peptide III is expected to be extremely air sensitive and adequate precautions may not have been taken in the sample handling.

Short sequences of peptides with the repeating subunits Cys-X-X-Cys can accommodate 4Fe-4S centers when used in ligand substitution reactions with preformed 4Fe-4S centers. The procedure for reconstitution, however, is more straightforward than the synthesis of 4Fe-4S analogues and provides a convenient route to the incorporation of iron-sulfur clusters in an apo-peptide. It is suggested here that these simpler reconstitution experiments be repeated in order to identify the nature of the iron-sulfur cluster which may be reconstituted. The demonstration that a primitive iron-sulfur peptide could be formed in aqueous solution would be a preliminary step toward answering the questions concerning the evolution of ferredoxins.

V. Other Experiments

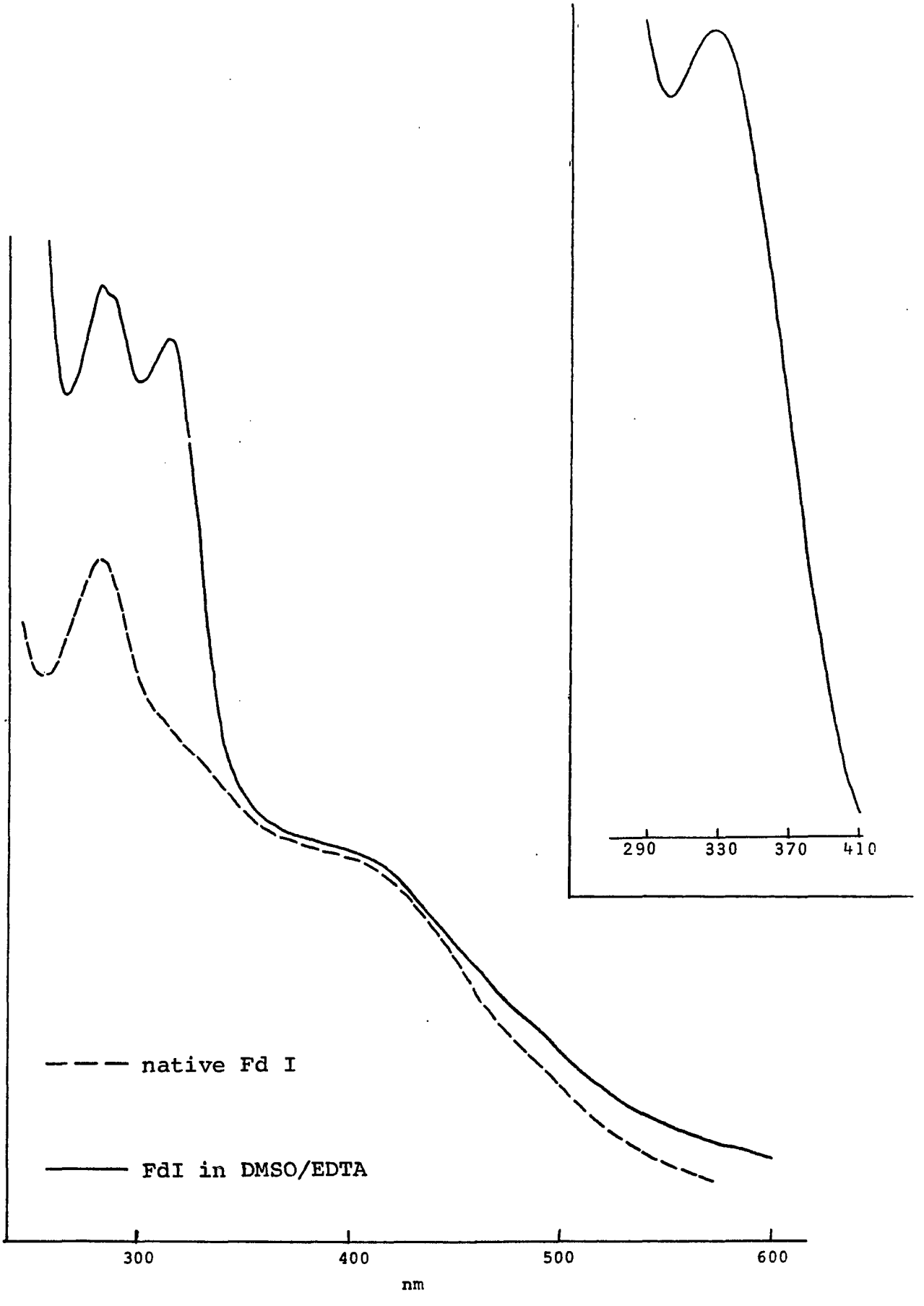
A. Influence of denaturants on *A. vinelandii* FdI

In the absence of the information provided by the completed x-ray analyses of *A. vinelandii* ferredoxin I, several sets of experiments were performed in an attempt to isolate an iron-deficient ferredoxin. In one set, solutions of Fd I were incubated with EDTA in buffer and EDTA in 60% DMSO. The Fd I in DMSO solutions required strict anaerobic handling in order to prevent the complete and rapid denaturation of the ferredoxin. Solutions were handled by syringe, through serum caps, in an oxygen free nitrogen atmosphere. The spectrum in Figure XXII indicates that iron is released from the ferredoxin under treatment overnight with EDTA in DMSO. The nature of the absorbance near 400 nm does not change suggesting that there is an 'all or none' loss of iron from both clusters in this ferredoxin. It should be noted, however, that no conclusive remarks based on the optical spectra of iron-sulfur centers should be made because of the similarities in the 400 nm region of all the protein spectra.

It was noted in the procedure to prepare apoferredoxin I that this ferredoxin is more stable to acid denaturation than *C. pasteurianum* ferredoxin. This observation suggested that one of the iron-sulfur centers may be more acid-labile than the other and that a ferredoxin containing only one center could be isolated from acid solution. It was found, however, that incubation of ferredoxin I at pH 3.8

Figure XXII. The optical spectrum of *Azotobacter vinelandii* ferredoxin I in DMSO/EDTA solution

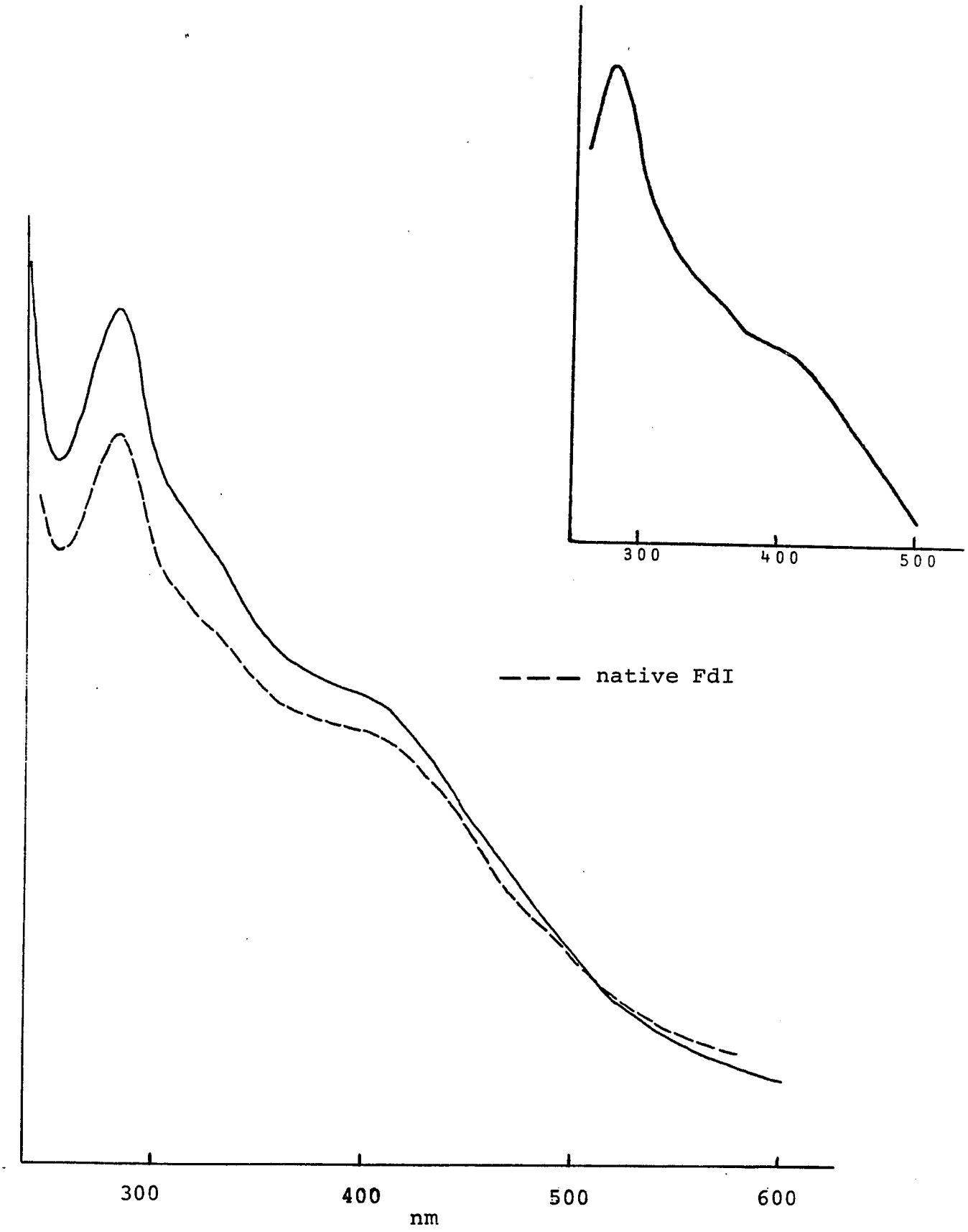
Inset: Optical spectrum of Fe^{III} in 80% DMSO


290
330
370
410
native Fd I
FdI in DMSO/EDTA
300
400
500
600
nm

probably resulted in an isoelectric precipitation based on the optical spectra in Figure XXIII. The similarities of iron-sulfur center optical spectra prevent, however, a definitve conclusion from these experiments. Epr experiments were undertaken to determine if one cluster per molecule had been removed but these were not completed because of an inability to reproduce the spectrum characteristic of oxidized *A. vinelandii* ferredoxin I required as a control. sample.

Figure XXIII. The optical spectrum of *Azotobacter vinelandii* ferredoxin I after acid treatment and removal of precipitate

Inset: Optical spectrum of supernatant from precipitation at pH 3.8 (as above). The supernatant was titrated back to pH 7.4.


300
400
500
native FdI
300
400
500
600
nm

Reconstitution of *Clostridium pasteurianum* ferredoxin from Deuterium Oxide

This procedure is included here as a foreword to Appendix I and describes the procedures omitted from the manuscript that follows.

A. Preparation of apoferredoxin

Approximately 10 mg *C. pasteurianum* ferredoxin (∿ 10 ml) in saturated ammonium sulfate was dialyzed against 4 l distilled water to remove $(NH_4)_2SO_4$. The protein was made 5% in trichloroacetic acid with solid TCA and incubated for one hour. The suspension was centrifuged at 33000 x g for fifteen minutes. The pellet was suspended and incubated in 5% TCA for 30 minutes and recentrifuged. The pellet was brought up in 5% $NaHCO_3$ and any insoluble material was removed by centrifugation. The solution was then dialyzed overnight in 4 l 0.01 M sodium acetate, then dialyzed against 4 l water with three changes, overnight. The solution was then lyophylized and the powder stored at 0^{o}.

B. Reconstitution

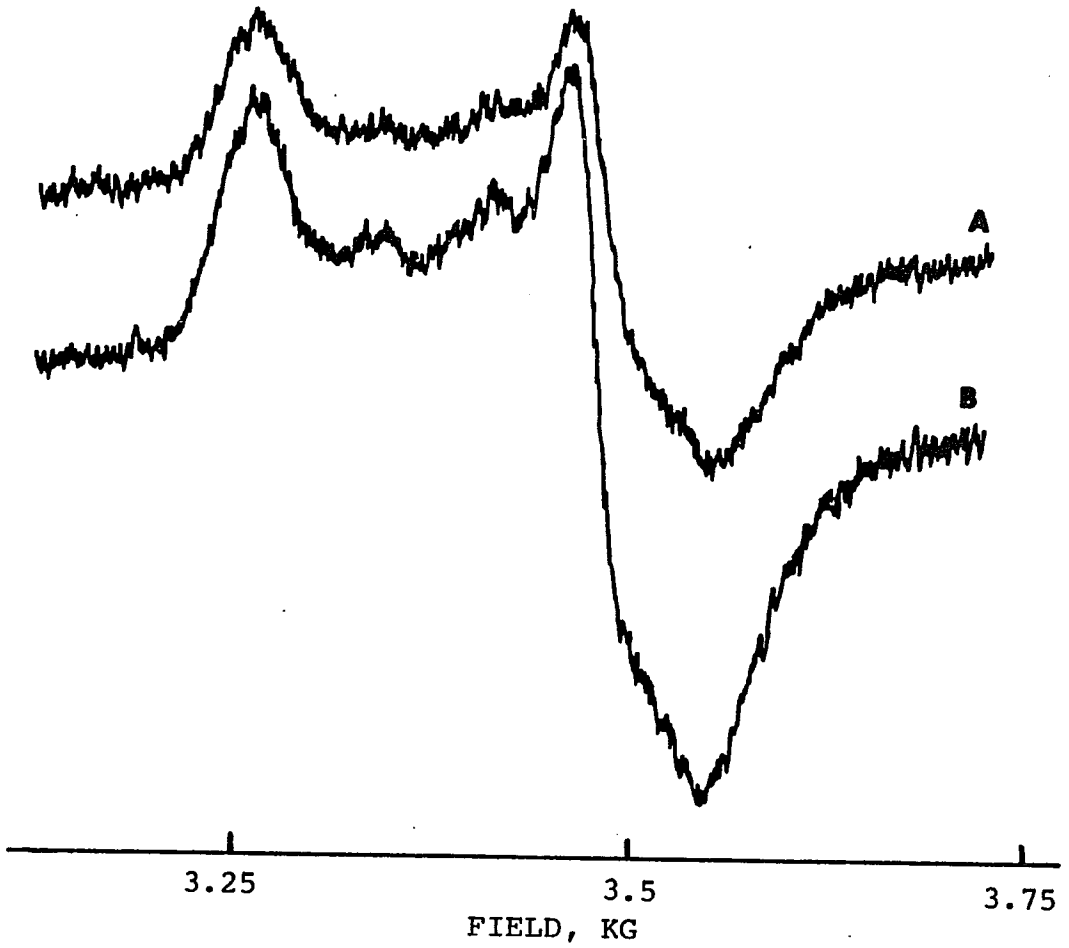
A solution containing 8 M d-4 urea, 0.07 M dithiothreitol and 0.125 M Tris-HCl, pH 8.2, and 3 mg apo-ferredoxin in 0.5 ml D_2O was incubated under an atmosphere of nitrogen for four days. To reconstitute, 0.12 ml 0.1 M ferrous ammonium sulfate and 0.12 ml 0.1 M sodium sulfide (both in D_2O) and 3 ml 0.035 M dithiothreitol in 0.1 M Tris-HCl (pH

7.4) in D_2O were added. This mixture was incubated for fifteen minutes under nitrogen. The mixture was diluted to 10 ml and applied to a DEAE column equilibrated with 0.1 M Tris-HCl (pH 7.4). The column was washed with 0.23 M NaCl in 0.1 M Tris-HCl (7.4) and the reconstituted ferredoxin was eluted with 0.5 M NaCl in 0.1 M Tris-HCl (pH 7.4) in D_2O. The fraction containing holoprotein was precipitated by making the solution 90% saturated in $(ND_4)_2SO_4$ and this precipitate was stored anaerobically at 5^{o}.

Please note that Figure XXIV is included also as an addendum to the Appendix because the epr spectra were not published in Biopolymers.

Figure XXIV. Epr spectra of *Clostridium pasteurianum* ferredoxin in H_2O and D_2O

Conditions: temperature, 13 K; gain, 630; modulation amplitude, 2.5 G; power, 2 mW; ν, 9.40 GHz; buffer, A: 50 mM K_2DPO_4/KD_2PO_4, pD, 8.07; B: 50 mM K_2HPO_4/KH_2PO_4, pH, 7.51


A
B
3.25
3.5
3.75
FIELD, KG

REFERENCES

1. Lovenberg, W., ed., (1977) Iron-Sulfur Proteins, Vols. 1, 2, 3. Academic Press, New York.

2. Yoch, D.C. and Carithers, R.P. (1979) Microbiol. Reviews (1979) 43: 384.

3. Brintzinger, H., Palmer, G., and Sands, R.H. (1966) Proc. Natl. Acad. Sci. USA 55: 397.

4. Johnson, C.E., Cammack. R., Rao, K.K., and Hall, D.O. (1971) Biochem. Biophys. Res. Commun. 43: 564.

5. Carter, C.W., Jr., Kraut, J., Freer, S.T., Xuong, Ng. H., Alden, R.A., and Bartsch, R.G. (1974) J. Biol. Chem. 249: 4212.

6. Carter, C.W., Jr., Kraut, J., Freer, S.T., and Alden, R.A. (1974) J. Biol. Chem. 249: 6339.

7. Freer, S.T., Alden, R.A., Carter, C.W., Jr., and Kraut, J. (1975) J. Biol. Chem. 250: 46.

8. Sweeney, W.V., Rabinowitz, J.C., and Yoch, D.C. (1975) J. Biol. Chem. 250: 7842.

9. Stout, C.D. (1979) Nature 279: 83.

10. Stout, C.D., Ghosh, D., Pattabhi, V., and Robbins, A. H. (1980) J. Biol. Chem. 255: 1797.

11. Sweeney, W.V. and Rabinowitz, J.C. (1980) Ann. Rev. Biochem. 49: 139.

12. Mortenson, L.E., Valentin, R.C. and Carnahan, J.E. (1962) Biochem. Biophys. Res. Commun. 7: 448.

13. Tanaka, M., Nakashima, T., Benson, A.M., Mower, H., and Yasunobu, K.T. (1964) Biochem. Biophys. Res. Commun. 16: 422.

14. Adman, E.T., Sieker, L.C., and Jensen, L.H. (1976) J. Biol. Chem. 251: 3801.

15. Yasunobu, K.T. and Tanaka, M. in Iron-Sulfur Proteins,

Vol.2, (Lovenberg, W., ed.) p. 40 (1973) Academic Press, New York.

16. Krasna, A.I. (1979) Enzyme Microb. Technol. 1: 165.

17. Holm, R.H. and Ibers, J.A. in Iron-Sulfur Proteins, Vol. 3, (Lovenberg, W., ed.) p. 247 (1977) Academic Press, New York.

18. Hong, J.S. and Rabinowitz, J.C. (1970) J. Biol. Chem. 245: 4982.

19. Lode, E.T., Murray, C.L., and Rabinowitz, J.C. (1976) J. Biol. Chem. 251: 1683.

20. Mathews, R., Charlton, S., Sands, R., and Palmer, G. (1974) J. Biol. Chem. 249: 4326.

21. Poe, M., Phillips, W.D., McDonald, C.C., and Lovenberg, W. (1970) Proc. Natl. Acad. Sci. USA 65: 797.

22. Hong, J.S. and Rabinowitz, J.C. (1970) J. Biol. Chem. 245: 4982.

23. Carter, C.W., Jr., Kraut, J., Freer, S.T., Alden, R.A., Sieker, L.C., Adman, E.T., and Jensen, L.H. (1972) Proc. Natl. Acad. Sci. USA 69: 3526.

24. Thompson, C.L., Johnson, C.E., Dickson, D.P.E., Cammack, R., Hall, D.O., Weser, V., and Rao, K.K. (1974) Biochem. J. 139: 97.

25. Emptage, M.H., Kent, T.A., Huynh, B.H., Rawlings, J., Orme-Johnson, W., And Munck, E. (1980) J. Biol. Chem. 255: 1793.

26. Herskovitz, T., Averill, B.A., Holm, R.H., Ibers, J.A., Phillips, W.D., and Weiker, J.F. (1972) Proc. Natl. Acad. Sci. USA 69: 2437.

27. Holm, R.H. and Ibers, J.A. in Iron-Sulfur Proteins, Vol. 3, (Lovenberg, W., ed.) pp. 205-281 (1977) Academic Press, New York.

28. Que, L., Jr., Anglin, J.R., Bobrik, M.A., Davison, A., and Holm, R.H. (1974) J. Am. Chem. Soc. 96: 6042.

29. Chen, J.S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta 371: 283.

30. Nakos, G. and Mortenson, L.E. (1971) Biochim. Biophys. Acta 227: 576.

31. Nakos, G. and Mortenson, L.E. (1971) Biochemistry 10: 2442.

32. Erbes, D.L., Burris, R.H., and Orme-Johnson, W.H. (1975) Proc. Natl. Acad. Sci. USA 72: 4795.

33. Gillum, W.O., Mortenson, L.E., Chen., J.S., and Holm, R.H. (1977) J. Am. Chem. Soc. 99: 584.

34. Rabinowitz, J.C. (1972) Methods Enzymol. 24: 431.

35. Covington, A.K., Paabo, M., Robinson, R.A., and Bates, R.G. (1968) Anal. Chem. 40: 700.

36. Stombaugh, N.A., Sundquist, J.E., Burris, R.H., and Orme-Johnson, W.H. (1976) Biochemistry 15: 2633.

37. Fee, J.A. Mayhew, S.G., and Palmer, G. (1971) Biochim. Biophys. Acta 245: 196.

38. Mizrahi, I.A., Wood, F.E., and Cusanovich, M.A. (1976) Biochemistry 15: 343.

39. Ingledew, W.J. and Ohnishi, T. (1980) Biochem. J.186: 111.

40. Ohnishi, T. (1976) Eur. J. Biochem. 64: 91.

41. Sobel, B.E. and Lovenberg, W. (1966) Biochemistry 15: 6.

42. Tagawa, K. and Arnon, D.I. (1968) Biochim. Biophys. Acta 153: 602.

43. Eisenstein, K.K. and Wang, J.H. (1969) J. Biol. Chem. 244: 1720.

44. Phillips, W.D. and Poe, M. in Iron-Sulfur Proteins, Vol. 2, (Lovenberg, W., ed.) p. 271, (1973) Academic Press, New York.

45. Dutton, P.L. and Wilson, D.F. (1974) Biochim. Biophys. Acta 346: 165.

46. Peisach, J., Orme-Johnson, N.R., Mims, W.B., and Orme-Johnson, W.H. (1977) J. Biol. Chem. 252: 5643.

47. Job, R.C. and Bruice, T.C. (1975) Proc. Natl. Acad. Sci. USA 72: 2478.

48. Shire, S.J., Hanania, G.I.H., and Gurd, F.R.N. (1974) Biochemistry 13: 2967.

49. Adman, E., Watenpaugh, K.D., and Jensen, L.H. (1975) Proc. Natl. Acad. Sci. USA 72: 4854.

50. Shire, S.J., Hanania, G.I.H., and Gurd, F.R.N. (1974) Biochemistry 13: 2974.

51. Sweeney, W.V. and McIntosh, B.A. (1979) J. Biol. Chem. 254: 4499.

52. Packer, E., Sternlicht, H., Lode, E., and Rabinowitz, J.C. (1975) J. Biol. Chem. 250: 2062.

53. Thauer, R.K., Kaufer, B., Zahringer, M., and Jungermann, K. (1974) Eur. J. Biochem. 42: 447.

54. Erbes, D.L., Burris, R.J., and Orme-Johnson, W.H. (1975) Proc. Natl. Acad. Sci. USA 72: 4795.

55. Purec, L., Krasna, A.I., and Rittenberg, D. (1962) Biochemistry 1: 270.

56. Kempner, W. and Kubowitz, F. (1933) Biochem. Z. 265: 245.

57. Kempner, W. (1933) Biochem. Z. 257: 41.

58. Carrell, H.L., Glusker, J.P., Job, R., and Bruice, T. C. (1977) J. Amer. Chem. Soc. 99: 3683.

59. Silverstein, R.M. (1975) Anal. Biochem. 63: 281.

60. Tsunoda, J.N., Yasunobu, K.T., and Whiteley, H.R. (1968) J. Biol. Chem. 243: 6262.

Study of the Influence of NH···S Hydrogen Bonds on the Reduction Potential in *Clostridium pasteurianum* 2(4Fe-4S) Ferredoxin Using Deuterium Exchange

WILLIAM V. SWEENEY and RICHARD S. MAGLIOZZO,
Department of Chemistry, City University of New York, Hunter College, New York, New York 10021

Summary

The effect of deuterium substitution of exchangeable hydrogen atoms on the reduction potential of *Clostridium pasteurianum* 2(4Fe-4S) ferredoxin has been studied. The studies were conducted to determine if NH···S hydrogen bonds to the iron–sulfur cluster are dominant in the mechanism of influence of the protein on cluster reduction potential, as has been proposed [Carter, C. W. (1977) *J. Biol. Chem.* **252**, 7802–7811]. Deuteration of the slowly exchangeable hydrogen atoms, however, yields essentially no shift in the reduction potential (-0.2 ± 0.8 mV), suggesting that NH···S bonds are not important modifiers of cluster reduction potential in this protein.

INTRODUCTION

One important aspect of iron-sulfur protein research is the origin of the reduction potential of 4Fe-4S clusters in these proteins. Such clusters appear in a variety of proteins, including high-potential iron–sulfur proteins (HiPIPs), which contain only one cluster, and low-potential, clostridial-type ferredoxins, which contain two clusters. The peptide in iron–sulfur proteins exerts an influence on the reduction potential of clusters, acting to shift $E_{1/2}$ to more positive values. The following four mechanisms of peptide influence on $E_{1/2}$ seem most likely: (1) polypeptide constraint of the geometry of the 4Fe-4S clusters; (2) electrostatic and dielectric effects[1]; (3) solvent or proton association with the clusters; (4) NH···S hydrogen bonds to the iron–sulfur clusters.[2] NH···S hydrogen bonds have been identified in the x-ray structures of both *Chromatium* HiPIP[3] and *Peptococcus aerogenes* 2(4Fe-4S) ferredoxin.[4] *P. aerogenes* ferredoxin exhibits extensive sequence homology to *Clostridium pasteurianum* ferredoxin[5] and has been reported to contain 15–18 NH···S hydrogen bonds.[4] It has been proposed that these bonds are the major mechanism of influence of the polypeptide on reduction potential.[2] An attempt to correlate the number of available hydrogen-bond donors with reduction potential in both proteins and model compounds has been reported,[2] from which an influence of about 650 mV was estimated for clostridial-type ferredoxins. Differences

Biopolymers, Vol. 19, 2133–2141 (1980)

0006-3525/80/0019-2133

in solvent composition, however, complicate those comparisons. A study by Hill et al.[6] indicates a smaller peptide influence, approximately 100 mV. NH···S hydrogen bonds would stabilize a reduced cluster relative to an oxidized one[7] and x-ray studies[3] show that the NH···S bonds in *Chromatium* HiPIP shorten by an average of 0.2 Å on reduction. Furthermore, it is observed that there are more NH···S bonds in *P. aerogenes* $2(4Fe\text{-}4S)^{2+;1+}$ ferredoxin than in *Chromatium* $(4Fe\text{-}4S)^{3+;2+}$ HiPIP.[2] The effect of deuteration of the slowly exchangeable protons on the $E_{1/2}$ of *C. pasteurianum* ferredoxin was investigated in an effort to establish experimental evidence for the influence of these bonds on the reduction potential in iron–sulfur proteins.

MATERIALS AND METHODS

Clostridium pasteurianum was grown and ferredoxin isolated according to the procedure of Rabinowitz.[8] Apoferredoxin was prepared according to the procedure of Hong and Rabinowitz[9] and reconstituted using a modification of a published procedure.[10]

Reduction potentials were determined using partially purified *C. pasteurianum* hydrogenase as described by Lode et al.[11] In the determination of the reduction potential in D_2O, deuterium gas was used, and a correction[12] was made for the standard reduction potential of the $2D^+/D_2$ couple (−3.4 mV). pD was determined by adding 0.41 to the reading obtained on a conventional pH meter with a glass electrode.[13] It was observed that D_2O-reconstituted material was spectrophotometrically identical to native ferredoxin, exhibiting the same spectral change on reduction.

Optical measurements were made using a Cary 219 uv–visible spectrophotometer. Proton-nmr spectra were obtained using a Bruker 270-MHz spectrometer at the NIH Regional Facility in New Haven, Conn. EPR spectra were obtained using a Varian V-4500 X-band spectrometer equipped with a Heli-Tran liquid helium transfer system (Air Products).

RESULTS

In order to separate the effects of deuteration on the reduction potential into those that arise from slowly exchanging protons and those that arise from rapidly exchanging protons and solvent effects, two separate types of experiments were performed. In the first type, apoferredoxin was incubated in D_2O for at least 72 hr to allow substitution of all exchangeable protons and then reconstituted from D_2O. Protein was removed from D_2O either by ammonium sulfate precipitation or ion-exchange chromatography, and the reduction potential was determined in H_2O solution. In this way, only the effects of slowly exchanging protons were observed. In a second type of experiment, ammonium sulfate-precipitated ferredoxin was dissolved in D_2O, and the reduction potential was measured immediately.

Thus, only effects arising from rapidly exchanging protons and solvent effects would be observed in this type of experiment.

The effect on $E_{1/2}$ of deuteration of the slowly exchanging protons is shown in Table I. For each determination, the $E_{1/2}$ of both a deuterium-exchanged sample and native ferredoxin were measured. Correction for differences in pH were made because of the pH dependence of the reduction potential of this protein.[11,14] The average difference in the pH-adjusted $E_{1/2}$ between the deuterium-exchanged sample and the control in these trials was −0.2 mV, with a probable uncertainty of 0.8 mV (95% confidence limits).

The effect on $E_{1/2}$ arising from deuteration of rapidly exchanging protons and solvent effects is substantially larger (Table II). The pH and pD conditions were chosen to give approximately the same degree of ionization of the buffer rather than the same H^+ and D^+ concentrations.[15] It is questionable whether a pH correction should be made in this experiment. The observed shift in the midpoint reduction potential without pH correction is 16 mV; with pH correction, 7 mV.

DISCUSSION

Isotope Effects

ND···S bonds are expected to have a lower zero-point vibrational energy than NH···S bonds and, therefore, a greater bond strength because of the larger mass of deuterium. Experiments indicate that the heat of sublimation of D_2O is 5% greater than that for H_2O.[16] Similarly, D_2O boils at a higher temperature than H_2O. The influence of hydrogen bonding on the midpoint reduction potential is equal to the difference in NH(D)···S bond strength between the oxidized and reduced states of clusters:

$$E^{H}_{rd} - E^{H}_{ox} = \Delta E^{H}$$

$$E^{D}_{rd} - E^{D}_{ox} = \Delta E^{D}$$

If a 5% increase in bond strength is assigned to ND···S bonds, then

$$\Delta E^{D} = 1.05\ (E^{H}_{rd} - E^{H}_{ox})$$

The thermodynamic deuterium isotope effect that will be observed when comparing native protein midpoint reduction potentials to those of deuterated derivatives is

$$\Delta E^{D} - \Delta E^{H} = 0.05\ (\Delta E^{H})$$

In other words, an increase in hydrogen-bond strength would be expected to shift the reduction potential by an amount proportional to the magnitude of influence NH···S bonds exert on the potential.

TABLE I
Effect of Deuterium Substitution of the Slowly Exchanging Hydrogen-Bonded Protons on Reduction Potential[a]

	Trial 1			Trial 2			Trial 3			Trial 4		
	$E_{1/2}$	pH	$E_{1/2}$ adj	$E_{1/2}$	pH	$E_{1/2}$ adj	$E_{1/2}$	pH	$E_{1/2}$ adj	$E_{1/2}$	pH	$E_{1/2}$ adj
D_2O exchanged	−411.4	7.44	−411.4	−407.5	7.41	−407.5	−410.7	7.46	−410.7	−403.0	7.38	−403.0
Native	−412.0	7.49	−411.2	−408.5	7.47	−407.6	−410.3	7.50	−409.7	−404.4	7.45	−403.3
$\Delta E_{1/2}$	—	—	−0.2	—	—	+0.1	—	—	−1.0	—	—	+0.3

[a] The midpoint reduction potentials (reported in millivolts) were adjusted for differences in pH (see text). The buffer in trials 1–3 was 50 m*M* potassium phosphate; in trial 4, the buffer was 100 m*M* Tris·HCl, with an approximate NaCl concentration of 0.4*M* resulting from DEAE chromatography (see Methods). Temperature, atmospheric pressure, and ionic strength (for trial 4) were not constants in different trials, so only differences in the same trial are directly comparable.

TABLE II
Effect of Solvent on the Reduction Potential[a]

	Trial 1			Trial 2		
	$E_{1/2}$	pH (D)	$E_{1/2}$ adj	$E_{1/2}$	pH (D)	$E_{1/2}$ adj
D_2O solution	−425.6	8.09	−425.6	−428.9	8.07	−428.9
H_2O solution	−409.9	7.53	−418.9	−412.4	7.53	−421.1
$\Delta E_{1/2}$	−15.7	—	−6.7	−16.5	—	−7.8

[a] The values $E_{1/2}$ adj (reported in millivolts) reflect an adjustment for differences in hydrogen-ion concentration. Reduction potential differences are shown for both the observed potentials and the pH-adjusted potentials, as discussed in the text. For both the H_2O and D_2O samples, 50 m*M* potassium phosphate buffer was used. Temperature and atmospheric pressure were not constant for different trials, and only differences within the same trial may be directly compared.

Slowly Exchanging Protons

Most of the slowly exchanging protons arise from NH···S bonds. Slow exchange occurs as a result of hydrogen bonding,[17] in conjunction with limited solvent accessibility. There are 15–18 NH···S and 8 NH···O hydrogen-bonded protons evident in the x-ray structure of *P. aerogenes* 2(4Fe-4S) ferredoxin.[4] Tritium exchange studies of *C. acidi-urici* 2(4Fe-4S) ferredoxin indicate 27 slowly exchangeable protons,[18] essentially as would be predicted from the x-ray results. The tritium exchange studies indicate that after 90 min, 19 of the original 27 protons remain unexchanged. Measurement of $E_{1/2}$ requires 60–90 min, and thus these exchange studies suggest that even if all of the hydrogen-bonded protons which exchanged in the first 90 min arose from NH···S bonds, about half of the NH···S protons would remain unexchanged. The x-ray structure clearly indicates that the NH···S protons have less solvent exposure than the NH···O protons. The fact that 12 protons remain unexchanged after 9 hr suggests that limited solvent access may be important for the exchange rate of some protons, increasing the probability that most of the protons unexchanged after 90 min are NH···S bonded.

Slowly exchangeable hydrogen-bonded NH protons exhibit nmr resonances in the region between 6 and 10 ppm.[19] Oxidized *C. pasteurianum* ferredoxin in H_2O solution was diluted with D_2O to 80% D_2O:H_2O (v/v), and the nmr spectrum was recorded at several time intervals (23°C, potassium phosphate buffer; D_2O, pD 7.9; H_2O, pH 7.9). Comparison was made of the 6–10-ppm region between these spectra and the spectra of ferredoxin in 100% D_2O and 90% H_2O:D_2O (v/v). While it is difficult to quantitate precisely the number of exchangeable resonances in each spectrum, it is estimated that 27 exchangeable hydrogen atoms remain unexchanged after 10 min. After 90 min, the maximum time required to complete a reduction potential measurement, roughly 15 exchangeable hydrogen atoms remain unexchanged. These estimates are in good agreement with the tritium exchange results of Hong and Rabinowitz.[18]

The results of the nmr experiments, in conjunction with the results from the tritium exchange studies, suggest that the deuterated derivative is stable

under the conditions of potential measurements and that at least half of the exchangeable amide donors remain deuterated based on the foregoing discussion. The fact that essentially no deuterium isotope effect was found ($\Delta E_{1/2} = -0.2 \pm 0.8$ mV) suggests that NH···S hydrogen bonds are minor influences on the midpoint reduction potential in this ferredoxin. For comparison, if NH···S hydrogen bonds exert a 100-mV influence on $E_{1/2}$, and deuterium substitution increases the bond strength by 5% (see discussion above), then a 5-mV shift in reduction potential would be predicted for the fully deuterated derivative.

Though the results strongly suggest a minor influence, they are not conclusive. The prediction that the shift in $E_{1/2}$ with deuterium substitution is directly related to the magnitude of the influence of NH···S bonds is based on the assumption that the effect of the deuterium isotope will be the same in both the oxidized and reduced states. While this is probable, there are circumstances under which it is not true. If all the potential wells for the NH···S bonds were broader in the reduced protein than in the oxidized protein, the change in hydrogen-bond strength on deuteration would be less in the reduced than in the oxidized state. Such an effect would lead to a negative shift in $E_{1/2}$, possibly canceling the expected positive shift from the formation of stronger bonds. However, there is no reason to expect such special circumstances.

Rapidly Exchanging Protons and Solvent Effects

It is possible that the sole mechanism of influence of the NH···S hydrogen bonds is through formation of new bonds in the reduced protein. The protons involved in these NH···S bonds would be rapidly exchanged in the oxidized protein and thus not detected in the experiments discussed above. Deuteration of these protons would lead to a positive shift in the reduction potential. To probe the effect of these hypothesized, newly forming hydrogen bonds, the protein was dissolved directly in D_2O and its reduction potential determined immediately. In this way, only the rapidly exchanging protons would be deuterated. However, this experiment indicated a negative shift in the reduction potential of 7–16 mV (Table II). This shift in $E_{1/2}$ is in the wrong direction to have arisen from ND···S bonds and probably arises from some other mechanism.

To determine if the shift in $E_{1/2}$ arose from a conformational change, nmr spectra of oxidized ferredoxin were recorded under the same solvent conditions used for the $E_{1/2}$ measurements. These spectra are shown in Fig. 1. The downfield resonances labeled β in this figure arise from protons on the β-carbon of cysteinyl residues. The resonances labeled α arise from α-carbon cysteinyl protons.[20] The shift in the α-carbon protons indicates that a protein conformation change may have occurred, but the close alignment of the β-carbon resonances suggests that any effect on the cluster conformation is, at most, very small.

To determine if a conformation change occurs in the reduced form of the

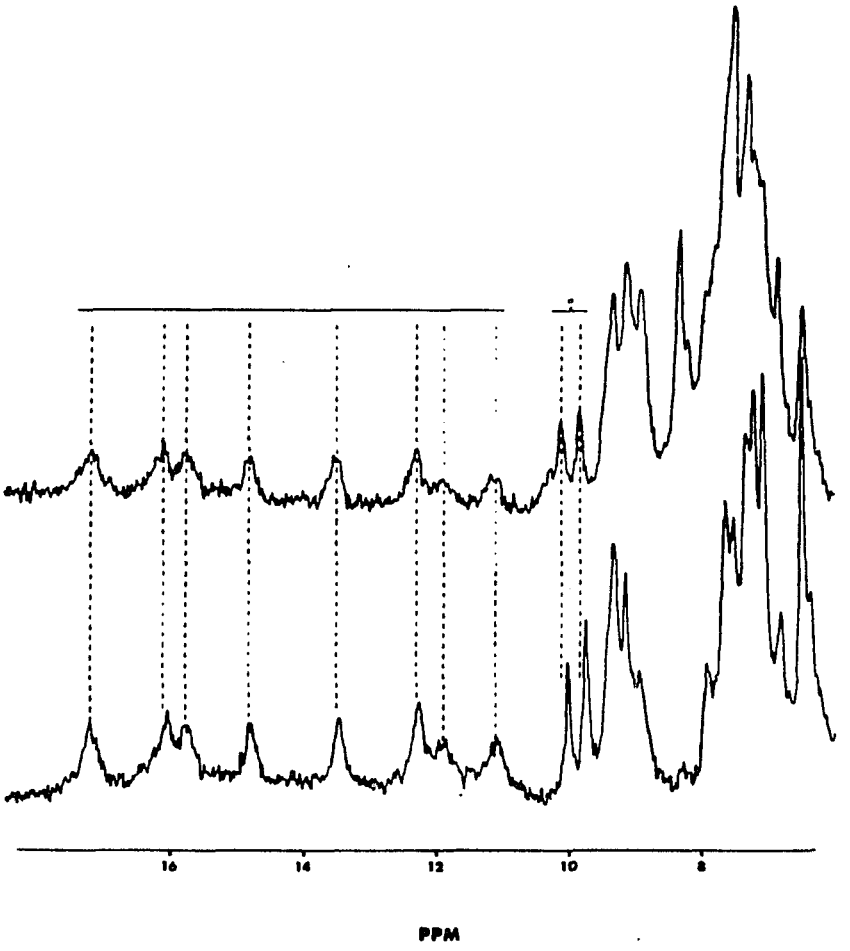


Fig. 1. 270-MHz ^{1}H-nmr spectra 22°C of oxidized *C. pasteurianum* ferredoxin in D_2O and H_2O solution, as described in Table II. Top spectrum: 5 mg in 0.14 ml (6 m*M*), 45 m*M* K_2HPO_4/KH_2PO_4 buffer, pH 7.51, with 10% D_2O to provide a reference lock for the spectrometer. Solvent suppression techniques were used to allow a high concentration of H_2O. Bottom spectrum: 5 mg in 0.14 ml (6 m*M*), 50 m*M* K_2DPO_4/KD_2PO_4 buffer, pD 8.07. Resonances marked β arise from protons bonded to cysteinyl β-carbons; those marked α arise from protons bonded to cysteinyl α-carbons.

protein, EPR spectra were recorded under the same conditions as the nmr spectra. Samples were slightly reduced using buffered sodium dithionite in order to allow direct comparison of the *g* values. EPR spectra of fully reduced *C. pasteurianum* 2(4Fe-4S) ferredoxin are complex and arise from interaction between the two paramagnetic 4Fe-4S clusters in the same molecule.[21] At slight degrees of reduction, however, the spectra arise predominantly from molecules containing only one reduced cluster, and direct comparison of the *g* values is therefore possible. The spectra exhibit identical *g* values, allowing the possibility of only very small conformational

differences. Therefore, although the α-proton resonances indicate a conformation change of the protein may have occurred, the β-proton resonance positions in the nmr spectra of the oxidized protein and the g values exhibited in the EPR spectra of the reduced protein suggest that the geometry of the 4Fe-4S clusters is not detectably altered in D_2O.

The most probable origin of the observed shift in $E_{1/2}$ in D_2O is direct interaction between the cluster and either solvent molecules or hydrogen ions. Such interaction is indicated both by the pH dependence of the midpoint reduction potential (R. S. Magliozzo and W. V. Sweeney, manuscript in preparation) and by linear electric field effect EPR experiments.[22]

CONCLUSION

The deuteration studies described here indicate that NH···S hydrogen bonds are not large influences on the reduction potential of the 4Fe-4S clusters in *C. pasteurianum* ferredoxin. A negative shift in the reduction potential observed for the protein in D_2O solution may arise from direct interaction of the 4Fe-4S clusters with solvent molecules or hydrogen ions.

This work was supported in part by Research Grant PCM76-21994 from the National Science Foundation and Research Grant GM 27382 from the National Institutes of Health. Acknowledgement for support is also made to the donors of the Petroleum Research Fund, administered by the American Chemical Society; the City University of New York, PSC-BHE Research Award Program; and the Southern New England High Field NMR Facility made possible by a grant from the Biotechnology Resources Program of the National Institutes of Health (RR-798).

References

1. Kassner, R. J. & Yang, W.(1977) *J. Am. Chem. Soc.* **99**, 4351–4355.
2. Carter, C. W. (1977) *J. Biol. Chem.* **252**, 7802–7811.
3. Carter, C. W., Jr., Kraut, J., Freer, S. T. & Alden, R. A. (1974) *J. Biol. Chem.* **249**, 6339–6346.
4. Adman, E., Watenpaugh, K. D. & Jensen, L. H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4854–4858.
5. Yasunobu, K. T. & Tanaka, M. (1973) in *Iron-Sulfur Proteins*, Vol. 2, Lovenberg, W., Ed., Academic, New York, pp. 27–130.
6. Hill, C. L., Renaud, J., Holm, R. H. & Mortenson, L. E. (1977) *J. Am. Chem. Soc.* **99**, 2549–2557.
7. Sheridan, R. P. & Allen, L. C. (1980) *Chem. Phys. Lett.* **69**, 600–604.
8. Rabinowitz, J. C. (1972) *Methods Enzymol.* **24**, 431–446.
9. Hong, J.-S. & Rabinowitz, J. C. (1967) *Biochem. Biophys. Res. Commun.* **29**, 246–252.
10. Hong, J.-S. & Rabinowitz, J. C. (1970) *J. Biol. Chem.* **245**, 4982–4987.
11. Lode, E. T., Murray, C. L. & Rabinowitz, J. C. (1976) *J. Biol. Chem.* **251**, 1683–1687.
12. *CRC Handbook of Chemistry and Physics*, 52nd ed. (1971–1972) Chemical Rubber Co., Cleveland, Ohio, p. D-115.

13. Covington, A. K., Paabo, M., Robinson, R. A. & Bates, R. G. (1968) *Anal. Chem.* **40**, 700–706.

14. Stombaugh, N. A., Sundquist, J. E., Burris, R. H. & Orme-Johnson, W. H. (1976) *Biochemistry* **15**, 2633–2641.

15. Bell, R. P. (1959) *The Proton in Chemistry*, Cornell U.P., Ithaca, N.Y.

16. Eisenberg, D. & Kauzmann, W. (1969) in *The Structure and Properties of Water*, Oxford UP, New York, p. 139.

17. Englander, S. W., Downer, N. D. & Teitelbaum, H. (1972) *Ann. Rev. Biochem.* **41**, 903–924.

18. Hong, J.-S. & Rabinowitz, J. C. (1970) *J. Biol. Chem.* **245**, 4995–5000.

19. Crespi, H. L., Kostke, A. G. & Smith, U. H. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1407–1414.

20. Packer, E. L., Sweeney, W. V., Rabinowitz, J. C., Sternlicht, H. & Shaw, E. N. (1977) *J. Biol. Chem.* **252**, 2245–2253.

21. Mathews, R., Charton, S., Sands, R. H. & Palmer, G. (1974) *J. Biol. Chem.* **249**, 4326–4328.

22. Peisach, J., Orme-Johnson, N. R., Mims, W. B. & Orme-Johnson, W. H. (1977) *J. Biol. Chem.* **252**, 5643–5650.

Received February 7, 1980
Accepted July 3, 1980