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STRUCTURAL AND OXIDATION-REDUCTION PROPERTIES OF (4-IRON \cdot 4-
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STRUCTURAL AND OXIDATION-REDUCTION PROPERTIES
OF \([4\text{Fe}-4\text{S}]^{3+},2^+\) FERREDOXINS

by
Craig Theodore Przysiecki

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF BIOCHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1984
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Craig Theodore Przybylo entitled Structural and Oxidation-Reduction Properties of $[4\text{Fe}-4\text{S}]^{3+,2+}$ Ferredoxins and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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Dedicated to JESS

to freedom

from

the aMeriCan way.
I would like to express my gratitude to Dr. Michael A. Cusanovich for his patience and support during the completion of this study. A special thanks goes to Dr. Terrance Meyer who generously supplied most of the HiPIPs used in this work. I am especially indebted to Dr. Gordon Tollin and members of his laboratory, as well as Dr. George Wilson's group, for the use of their laboratory equipment and many helpful discussions.
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Many biologically important electron transfer reactions involve iron-sulfur centers and flavosemiquinones. Laser flash photolysis and stopped-flow spectrophotometry has been utilized to investigate factors influencing transient kinetic oxidation of free and protein-bound flavosemiquinones by various types of oxidants, but primarily high potential iron-sulfur proteins (HiPIP) and rubredoxin classes of Fe-S proteins.

Studies of free flavosemiquinone analog oxidation by Fe-containing redox proteins and inorganic oxidants have found the reactivity of the anionic flavosemiquinone to be greater than that for neutral flavosemiquinone.

The second order rate constants of oxidation of various flavosemiquinone analogs by either redox proteins or non-biological oxidants correlate with the difference in redox potential of the reactants according to the Marcus exponential equation. For the protein-flavin analog reactions, deviations from the theoretical Marcus curve are interpreted in terms of the effects of the different exocyclic substitutions on intrinsic anion semiquinone reactivity.

Electrostatic effects on FMN and C. pasteurianum flavodoxin semiquinone oxidation by HiPIP can be quantitated yielding the rate constant at infinite ionic strength (k∞) and the charge product for
reaction. The magnitude of the electrostatic effects are larger for flavodoxin semiquinone oxidation than for FMN semiquinone oxidation which is consistent with the larger electrostatic charge for flavodoxin.

The $k_\text{obs}$ values obtained from the electrostatic analysis for FMN semiquinone and flavodoxin semiquinone oxidation indicate a dependence on the redox potential difference between the reactants ($\Delta E$). The $\Delta E$ effect is larger in magnitude for the flavodoxin reaction than for the FMN reaction.

Deviations from the theoretical curves for the FMN and flavodoxin reactions suggest that factors other than redox potential and electrostatics, such as sterics, could be having an important influence on reactivity. The results suggest that electrostatics, redox potential, and perhaps sterics could have an important role in determining the biological specificity of protein/protein redox reactions.

\textit{C. pasteurianum} rubredoxin forms a 1:1 complex with spinach ferredoxin:NADP+ reductase (FNR) at low ionic strengths. The reduction of Rd$_{\text{ox}}$ by lumiflavin semiquinone is unaltered in the complex. Evidence is presented for a rapid ($2 \times 10^3 s^{-1}$) intracomplex electron transfer from FNR semiquinone to oxidized rubredoxin.
INTRODUCTION

The biological function of Fe-S centers (Figure 1) within simple and complex iron-sulfur proteins is that of single electron transfer. In many instances, the electron transfer, either intramolecular or intermolecular, occurs between Fe-S sites and flavin cofactors. During these electron transfer reactions, direct and indirect evidence suggests involvement of the flavosemiquinone as an intermediate (for a review see Edmondson and Tollin 1983).

An example of intramolecular Fe-S center/flavin interaction is found in the dimeric trimethylamine dehydrogenase which contains a single 6-S-cysteinyl FMN flavin and one [4Fe-4S]^{2+,1+} cluster per monomer (Lim et al. 1982). The slow, apparently catalytically essential electron transfer from the substrate-reduced flavohydroquinone to the oxidized cluster leads to the formation of an anionic flavosemiquinone which is spin-coupled to the reduced cluster (Steenkamp and Beinert 1982).

Many examples of intermolecular Fe-S/flavosemiquinone electron transfer are part of multiple redox protein systems of the type:

\[
\text{NAD(P)H} \quad \xrightarrow{2e^-} \quad \text{FLAVOENZYME} \quad \xrightarrow{2(1e^-)} \quad \text{FE-S PROTEIN.}
\]

In this generalized redox scheme, flavoenzymes act as obligatory intermediaries between nicotinamide coenzymes and Fe-S proteins. During flavoenzyme catalysis, the obligatory two and one-electron transferring modes of nicotinamides and Fe-S proteins, respectively,
Figure 1. Atomic arrangement within recognized Fe-S centers as determined by X-ray crystallography (from Cammack 1980, with permission).
necessitates the involvement of the flavin semiquinone. Specific examples utilizing the above reaction proceeding to the right are found in the adrenal mitochondrial steroid hydroxylation system and the omega-hydroxylation system of *Pseudomonas oleovorans*. In these systems, the flavoenzyme-reduced Fe-S proteins, adrenodoxin and rubredoxin, respectively, directly provide the reducing equivalents to the hydroxylases of their systems. Alternatively, light-driven NADP+ reduction in plants uses the above redox scheme proceeding to the left with reduced ferredoxin transferring its electron to the chloroplast flavoenzyme, ferredoxin:NADP+-reductase (FNR). Although electron transfer in the above systems occurs intermolecularly, i.e. Fe-S sites and flavin cofactors reside in different polypeptide chains, the Fe-S protein and flavoenzyme are strongly associated in 1:1 complexes at low ionic strength (Kamin et al. 1980).

The establishment of structure/function relationships for electron transfer reactivity between flavin coenzymes and Fe-S proteins is of current research interest (Lambeth et al. 1980, Light and Walsh 1980, Batie and Kamin 1981, Zanetti et al. 1983). Of the simple Fe-S proteins, the [4Fe-4S]^{3+,2+} ferredoxin or high potential iron-sulfur protein (HiPIP) and rubredoxin (Rd) classes possess several advantages for use in detailed studies of Fe-S/flavin reactivity. As a class, HiPPIPs and Rds can be easily isolated and purified, being unusually stable at room temperature and ambient oxygen tensions. The $E_{m,7}$ values of HiPIP and Rd are thermodynamically favorable for reduction by both free and protein-bound flavosemiquinones. In addition, three dimensional structures are known for at least one member from each
class (Carter et al. 1974a, Adman et al. 1977, Watenpaugh, et al. 1979) as well as the primary structures of additional examples (Vogel et al. 1977, Tedro et al. 1981). An interesting structural distinction between HiPIP and Rd is the solvent accessibility of their Fe-S centers. The [4Fe-4S] cluster in HiPIP is relatively buried with the closest cluster surface to protein surface contact distance being four to five angstroms (Carter et al. 1974a); while the single iron center of Rd, including at least one of the cysteiny1 sulfurs, lies close to the protein surface (Watenpaugh et al. 1979). In the case of the HiPIP class, examples exist which differ in several important properties such as redox potential, net protein charge, extent of sequence similarity and size. This range of properties provides an opportunity for a more rigorous examination of flavosemiquinone reactivity, as well as structure/function relationships of HiPIP reduction.

Of the possible flavin redox partners for reactivity studies, the use of free flavins and the flavin-containing proteins, flavodoxin and ferredoxin:NADP+-reductase, can provide detailed complementary information. The availability of free flavin analogs with specific exocyclic substitutions that vary in charge, electronic, and steric properties should allow for investigating the effects of such changes on flavosemiquinone reactivity. The more advanced state of knowledge of the physico-chemical properties of free flavosemiquinones, as compared to those which are protein-bound, present a clear advantage for mechanistic interpretation. However, since the possibility of many molecular orientations of free flavins with Fe-S sites exists, complementary studies of a structurally-defined protein bound
flavosemiquinone with its geometrically-constrained orientation should prove informative. In this regard, flavodoxins are a good candidate in that they are structurally the most intensively studied class of flavoproteins. Currently, the three-dimensional structures of three flavodoxins have been in part or completely elucidated, including the structure of the thermodynamically-stabilized neutral flavosemiquinone redox state (for reviews see Mayhew and Ludwig 1975, Adman 1979). Precursor adduct formation is probably an important regulatory feature of in vivo electron transfer processes by controlling reaction partner recognition. Both computer modeling studies (Salemme 1976, Poulos and Kraut 1980, Poulos and Mauk 1983, Matthew et al. 1982) and experimental evidence (Foust et al. 1969, Smith et al. 1980, Simonsen et al. 1982) suggest that electrostatic interactions between redox proteins are important for complexation. Although FNR and Rd do not interact physiologically, reactivity studies of this system are of interest, since, like many biologically important reaction systems, the FNR/Rd system, forms a 1:1 complex at low ionic strength (Foust et al. 1969). Availability of a high resolution three-dimensional structure (Watenpaugh et al. 1979) of C. pasteurianum Rd and the possibility of structural information on FNR becoming available in the near future (Karplus and Herriott 1982) provides a useful system with which to initiate studies on the electron transfer kinetics of 1:1 flavoprotein reductase:Fe-S protein complexes.
HiPIP

Sources and Function

Since the first HiPIP was isolated from the photosynthetic purple sulfur bacterium, *Chromatium vinosum* (Bartsch 1963), eleven other sources for the protein have been identified. The thirteen additional sources include seven photosynthetic purple sulfur bacteria: *Chromatium gracile*, *Chromatium warmingii* (Wermter and Fischer 1983) *Thiocapsa roseopersicina*, *Thiocapsa pfennigii*, *Ectothiorhodospira halophila*, *Ectothiorhodospira shaposhnikovii* (Kusche and Truper 1984) and *Ectothiorhodospira vacuolata*; five photosynthetic non-sulfur purple bacteria: *Rhodopseudomonas gelatinosa*, *Rhodopseudomonas globiformis*, *Rhodospirillum tenue* strains 2761 and 3761, *Rhodomicrobium vannielli*; and one non-photosynthetic, halophilic, denitrifying bacterium: *Paracoccus sp* (for sources not referenced see Tedro et al. 1981 and Meyer 1982). The three *Ectothiorhodospira sps.* are apparently unique in that they possess two iso-HiPIP forms (Meyer et al. 1983, Kusche and Truper 1984). HiPIPs form a family of bacterial proteins distinct from other [4Fe-4S] ferredoxins based on apparent amino acid sequence homology, primarily of the coordinating cysteine residues. At present, ten of the seventeen examples of these monomeric 63-85 amino acid single polypeptides have been sequenced (Table 1). Thus, with the three-dimensional structure of *C. vinosum* HiPIP available, (cf. Carter

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Source: a

1 - C. vinosum, 2 - T. roseopersicina, 3 - C. gracile, 4 - T. pfennigii, 5 - Rp. gelatinosa,
6 - Paracoccus sp., 7 - E. halophila iso-2, 8 - E. halophila iso-1, 9 - Rs. tenue 3761,
10 - Rs. tenue 2761
1977a), substantial structural information exists to interpret kinetic results obtained using this class of [4Fe-4S] ferredoxins.

The exact functional role of HiPIP in electron transport remains obscure. Nevertheless, it has been suggested that \textit{C. vinosum} HiPIP may be involved in light-driven electron transport (Evans et al. 1974). More recently, \textit{C. vinosum} HiPIP has been found to act as a good electron acceptor for a thiosulfate-oxidizing enzyme partially purified from \textit{C. vinosum} (Fukamori and Yamanaka 1979).

The [4Fe-4S] Redox States

X-ray crystallographic studies have shown the cubane-like [4Fe-4S] cluster of reduced \textit{C. vinosum} HiPIP to be nearly identical to the two equivalent clusters found in oxidized \textit{Peptococcus aerogenes} 2[4Fe-4S] ferredoxin (Carter et al. 1972). Despite this similarity in prosthetic group structure, \textit{C. vinosum} HiPIP reversibly transfers a single electron at an oxidation-reduction potential of +350 mV (Bartsch 1978); while the oxidation-reduction potential of the two clusters of \textit{P. aerogenes} is -427 mV (Stombaugh et al. 1976). This drastic difference in oxidation reduction potential between these two proteins has been reconciled by the proposal that the clusters within these proteins can exist in three different oxidation states. This explanation has been referred to as the three-state hypothesis by Carter et al. (1972). The oxidation levels obtainable by the cluster are 3+, 2+, 1+, each differing by one electron, and represent the net charge of the cluster, excluding the cysteiny1 sulfur charge contributions. In terms of formal iron valences only, the 3+, 2+, and
1+ states correspond to [Fe(III)$_3$Fe(II)$_1$], [Fe(III)$_2$Fe(II)$_2$], and [Fe(III)$_1$Fe(II)$_3$], respectively (Herskovitz et al. 1972). In _C. vinosum_ HiPIP, the cluster is restricted, under native conditions, to the 3+, 2+ levels being oxidized from the diamagnetic 2+ level to the paramagnetic 3+ level ($g_{\text{avg}} < 2$). In contrast, the clusters in _P. aerogenes_ are reduced from the common paired-spin state 2+, to the paramagnetic 1+ oxidation level ($g_{\text{avg}} > 2$; cf. Carter et al. 1972).

Differences in the cluster binding cavities are thought to constrain the [4Fe-4S] clusters in these proteins to their respective oxidation levels. In favor of this hypothesis, HiPIP can be reduced to the normally unobtainable 1+ oxidation level, termed super-reduced HiPIP, upon partial protein unfolding in 80% DMSO (Cammack 1973). The protein structural features responsible for this thermodynamic prevention of super-reduction in native HiPIP are not known in detail (cf. Sweeney and Rabinowitz 1980). Nevertheless, it has been suggested that the differential exposure of the cluster to solvent (Kassner and Yang 1977), geometrical or electronic distortion of the cluster (Carter 1977b, Laskowski et al. 1978), and differential stabilization of cluster charge by NH•••S hydrogen bonds (Sheridan et al. 1981) may be involved.

Carter (1977b) has proposed that the differences in cluster surroundings may arise from tyrosine side chain interactions with an inorganic sulfur ($S^*_3$) of the cluster (Figure 2). These tyrosine/cluster interactions within _C. vinosum_ HiPIP and _P. aerogenes_ 2[4Fe-4S] Fd are from opposite sides of an effective mirror plane of the cluster (see HiPIP: _C. vinosum_ HiPIP Structure) and, along with
other polypeptide interactions, place the clusters into diastereomeric environments. A pair of bands of opposite ellipticity in the visible circular dichroism (CD) spectra of one reduced HiPIP, *G. vinosum* (negative maxima at 348 and 394 nm) and of an oxidized ferredoxin (positive maxima at 325 and 425 nm), structurally similar to *P. aerogenes*, were pointed out by Carter (1977b), as experimental support for this proposal. In addition, conditions which can generate super-reducible HiPIP result in striking changes in the two negative bands of reduced HiPIP at 348 nm and 394 nm, as well as a positive band at 458 nm, although the reversibility of these changes was not demonstrated (Carter 1977b).

Recent experimental evidence superficially suggests NH···S hydrogen bonds are not important modifiers of cluster oxidation-reduction potential (Sweeney and Magliozzo 1980). This study examined the effect of deuterium substitution of slowly exchanged protons on the oxidation-reduction potential of *Clostridium pasteurianum* 2[4Fe-4S] ferredoxin. The slowly exchanged protons were assumed to represent the protons of backbone amide hydrogen bonded to oxygen and the sulfur atoms of the cluster, as well as protons of low solvent accessibility. It was predicted, because of the greater strength of the deuterium substituted NH···S hydrogen bond, that an increase in redox potential would occur, contrary to the experimental findings. However, the authors did point out that if the potential energy wells for the NH···S bond were broader in the reduced than in the oxidized protein, no effect or the opposite effect might be observed. Others have suggested this special situation could conceivably apply to hydrogen bonding in
Fe-S proteins (Sheridan et al. 1981). Thus, the question of the influence of NH-H S hydrogen bonds on the redox properties of Fe-S proteins remains open to further experimental scrutiny.
Figure 2. Unique orientation of the [4Fe-4S] cluster and its cysteiny1 ligands (From Carter 1977b, with permission).

Fe atoms are represented by black circles and have same numbering as the cysteiny1 ligands (double circles) to which they are ligated. Inorganic sulfurs (S⁻) are indicated by open circles. Fe-S⁺ bonds are shortened by tetragonal distortion, indicated by an extra line, and are parallel to 4 axis. Trigonal distortion of cysteine ligation indicated by 3-axis.
Figure 2. Unique orientation of the [4Fe-4S] cluster and its cysteiny1 ligands (From Carter 1977b, with permission).
A recent proton nuclear magnetic resonance ($^1$H-NMR) study of *C. vinosum* and *R. gelatinosa* HiPIPs has led to discovery of two very high field hyperfine-shifted resonances (about -30 ppm) which may be unique to [4Fe-4S] ferredoxins using the 3+,2+ cluster oxidations levels (Nettesheim et al. 1983). The protons possibly originate from other than cysteiny1 ligand protons, perhaps from the protons of the strictly conserved TYR-19 or backbone NH protons hydrogen bonded to sulfur atoms of the cluster (Nettesheim et al. 1983). Strong electronic coupling between TYR-19 and the cluster has been suggested from movements observed within a complex TYR-19 hydrogen bonding network observed in crystallographic studies of oxidized and reduced HiPIP (Carter et al. 1975, see HiPIP: *C. vinosum* HiPIP Structure). These upfield shifted resonances, when more fully understood, should lead to a better understanding of structural factors involved in the restriction of the 3+,2+ oxidation levels to the HiPIP class.

**Redox Potential**

Once a pair of cluster oxidation states are determined by the protein moiety, the polypeptide presumably also controls the redox potential at which electron transfer occurs. The redox potential associated with the 3+,2+ oxidation levels at present appears to be positive, but can vary widely, from +50 to +450 mV (Meyer et al. 1983). An Fe-S protein previously thought to contain a low negative potential [4Fe-4S] cluster using the 3+,2+ oxidation levels, and thus disputing the validity of the above correlation (Sweeney et al. 1975), has been shown by spectroscopic and structural studies to instead possess a
novel [3Fe-3S] cluster (Emptage et al. 1980, Stout et al. 1980). The specific structural interactions of the protein moiety with the [4Fe-4S] cluster which affect the wide range of redox potentials exhibited by the HiPIP class are not well understood. Nevertheless, the redox-linked protonation state of the relatively distant (10 A) HIS-42 is suggested to have a small influence on the $E_{m,7}$ value of the clusters in *R. gelatinosa* and *C. vinosum* HiPIPs (Nettesheim et al. 1980).

In another class of redox proteins, the $c$-type cytochromes, it is proposed that modulation of the redox potential can be better described by dynamic, vibrational structure differences between the two oxidation states of these proteins, rather than static structural differences (Salemme 1979). To this end, solution spectroscopic studies of the $3+,2+$ oxidation levels of the [4Fe-4S] cluster merit discussion (for a review see Sweeney and Rabinowitz 1980).

The [4Fe-4S]$^{3+,2+}$ Center and HiPIP Spectroscopic Properties

The properties of the electronic absorption spectrum of each HiPIP are used to measure the concentration, oxidation state, and purity of the isolated proteins. The absolute visible absorption spectra of the reddish-brown colored oxidized ($\lambda_{\text{max}} \approx 450, 400, \text{and} 320 \text{ nm}$) and green-colored reduced ($\lambda_{\text{max}} \approx 390 \text{ nm}$) redox states of these proteins are very similar among the various sources (Bartsch 1978; Figure 3). The bands in the visible region are suggested to arise from charge-transfer type transitions ($S \rightarrow Fe$), with absorbance increasing with decreasing wavelength, and the oxidized state having a greater absorbance than the reduced state (Dus et al. 1967; Aizman and
Case 1982). In the near ultra-violet region the reduced state exhibits a slightly greater absorbance than the oxidized state, with the cluster presumably contributing to the absorbance in this region. It is in this wavelength region that the major differences between the various sources occur, primarily consequent to differences in aromatic amino acid content (Bartsch 1978).

The presence of a prominent 300 nm negative band, and two less resolved bands at 290 and 280 nm, in the oxidized-minus-reduced near UV difference spectrum of many HiPIPs (Figure 3) have been attributed to changes in the microenvironmental hydrophobicity of either TRP-76 and/or TRP-80, which are both highly conserved (Nettesheim et al. 1980). Perturbation difference spectroscopic studies as a function of pH (apparent pKa,s are 6.9 and 6.7 for the reduced and oxidized protein, respectively), and use of the histidine-selective modification reagent diethylpyrocarbonate with oxidized and reduced \textit{C. vinosum} HiPIP, also produce difference spectra with bands at approximately 300, 290, and 280 nm in the near UV and also in the visible region. These features of the difference spectra were rather convincingly interpreted as resulting from the deprotonation or modification of the sole HIS-42 of \textit{C. vinosum} HiPIP. In conjunction with kinetic studies of the pH dependence of \textit{C. vinosum} by ferricyanide (see HiPIP: Kinetics), and of ferricyanide oxidation of the diethylpyrocarbonate-modified HiPIP, it was further suggested that deprotonation or modification of HIS-42 destabilizes the reduced cluster, perhaps through disruption of a hydrogen bond, resulting in a faster rate of oxidation (Nettesheim et al. 1980).
Figure 3. Absorption spectra of Chromatium vinosum and Rhodopseudomonas gelatinosa high potential iron-sulfur proteins (HiPIP). Reprinted from Dus et al. 1967, with permission.
The four irons and inorganic sulfurs in the C. vinosum [4Fe-4S]^{2+} cluster can be described as having geometrical properties of two distorted concentric interpenetrating iron and inorganic sulfur tetrahedrons (Carter 1977b). The tetragonal distortion takes the form of compression of four $S^*-Fe$ bonds between atoms of two opposite faces of the cluster, parallel to the $T$ axis (Figure 2). Similar and more precisely determined symmetry elements have been observed in isoelectronic cluster analogs, suggesting this tetragonal distortion is an intrinsic property of the cluster (Averill et al. 1973). Additional distortions of the cluster are discussed in the section, C. vinosum HiPIP Structure on page 20. The iron atoms of the reduced cluster are equivalent on the basis of magnetic susceptibility measurements, despite the presence of mixed valence states (i.e. Fe(II) and Fe(III)), suggesting that the iron atoms are strongly antiferromagnetically exchange-coupled (Antanaitis and Moss 1975). The residual paramagnetism of the reduced cluster arises from thermal populations of low-lying excited spin states. $^1$H-NMR (294 K) studies have shown that this paramagnetism results in three hyperfine-shifted resonances at 12.6, 16.1, and 16.5 ppm, all of which exhibit anti-Curie temperature dependencies. The resonances have been tentatively assigned to the beta-CH$_2$ protons of the cysteinyl ligands (Phillips et al. 1970a).

Upon oxidation of C. vinosum HiPIP, the paramagnetic [4Fe-4S]$^{3+}$ cluster more closely approaches tetrahedral symmetry by contracting along certain dimensions (see HiPIP: C. vinosum HiPIP Structure), which is consistent with the greater Fe(III) character of the cluster (Carter 1977a). Despite this indication of nearly equivalent iron atoms from
the X-ray crystal structure, numerous solution spectroscopic studies suggest a substantial uneven distribution of unpaired electron spin. The existence of both Curie and anti-Curie temperature dependencies of the nine hyperfine-shifted cysteiny1 beta-CH$_2$ proton resonances suggests the presence of asymmetric spin density (Phillips et al. 1970a, Nettesheim et al. 1983). However, the finding of the extremely upfield hyperfine-shifted resonances in C. vinosum and Rp. gelatinosa HiPIP (see HiPIP: [4Fe-4S] Redox States) questions the assignment (cysteiny1 or noncysteiny1 protons) and/or the origin (dipolar or contact) of hyperfine-shifted resonances in both oxidized and reduced HiPIP (Nettesheim et al. 1983).

The electron paramagnetic resonance (EPR) X-band spectrum ($\leq$ 35 K) for oxidized C. vinosum HiPIP shows the superposition of two signals of equal weight. One signal is an axial signal with $g_\alpha = 2.12$ and $g_1 = 2.04$; the other is a rhombic signal with $g_1 = 2.087$, $g_2 = 2.055$, and $g_3 = 2.040$. This result has been interpreted as evidence for the unequal distribution among the irons of the unpaired electron formally associated with Fe(II) (Antanaitis and Moss 1975). Since the time of this extensive EPR study of C. vinosum HiPIP, the EPR spectra of Rp. gelatinosa (Blum et al. 1978), Rs. tenue (strain not given) (Sands 1979), E. halophila iso-2 (Cammack 1979) and T. roseopersicina (Zorin and Gogotov 1984) HiPIPs have been reported. A comparison of the five EPR spectra indicates the rhombic signal is peculiar to C. vinosum HiPIP, with the other HiPIPs exhibiting very similar axial signals with very little, if any, rhombic signal. A more recent EPR study of C. vinosum HiPIP suggests the rhombic component, which is now estimated to
represent approximately 15% of the total EPR intensity, is induced by freezing and presumably caused by protein association that results in spin-spin coupling. Simulations of the Mossbauer spectrum of oxidized *C. vinosum* HiPIP support the existence of a species in approximately 15% abundance that is unlike the remainder of the oxidized clusters (Goldsmith et al. unpublished results cited in Sands 1979). Thus, at present, there appears to be no EPR evidence for the inequivalence of the irons of the oxidized cluster.

However, evidence from electron nuclear double resonance (ENDOR) and Mossbauer studies still gives strong support for a model of the oxidized cluster as consisting of two pairs of iron atoms, each atom of the pair being equivalent (Moss et al. 1968, Anderson et al. 1975). Resonance Raman studies of Fe-S proteins have been severely limited by technical problems. However, based on presently available spectra, it has been concluded that no large splittings are observed for *C. vinosum* HiPIP for the nontotally symmetric modes which would be indicative of structural asymmetries in the cluster binding site. The detection and interpretation of small splittings will require better quality spectra. Also of interest is the fact that the breathing modes of the cluster are invariant to oxidation state; while shifts in the terminal Fe-SR frequencies are consistent with the strengthening of these bonds upon oxidation (Spiro et al. 1982).

*C. vinosum* HiPIP Structure

The C-terminal segment of the polypeptide chain binds (Cys 43, 46, 63, and 77) and insulates the cluster from the solvent by creating
a highly conserved hydrophobic binding cavity (for a review see Carter 1977a). The closest approach to the solvent is at a hydrophobic patch where the sulfur of CYS-46 is blocked from the solvent by THR-81 and PHE-48 and an inorganic \( S_4^* \) (Figure 2) is separated from the solvent by LEU-65 (Adman 1979). This solvent inaccessibility of the cluster has been emphasized in solution studies by studying electron spin echo decay, using pulsed EPR techniques which indicate no access of \( D_2O \) to the Fe-S center (Peisach et al. 1977).

As with most simple Fe-S proteins, HiPIP lacks substantial amounts of extended secondary structural elements. The hydrophobic cavity that binds the cluster is formed from a C-terminal segment (43-80) and a short amino terminal segment. The C-terminal segment contributes two anti-parallel beta-sheet structures containing cysteines 63 and 77 and two linked hair-pin turns which contain cysteine residues 43 and 46. The remaining portion of residues 1-42 folds upon the cluster binding region of the protein. Although the X-ray crystal structure suggests two short alpha-helical segments (~12% of the total polypeptide), no alpha-helical contribution to the far UV CD spectrum of \( C. vinosum \) HiPIP has been observed (Flatmark and Dus 1969).

The orientation of the cysteiny1 coordination creates a further geometric asymmetry in the reduced cluster (see section on [4Fe-4S]^{3+,2+} Center and HiPIP Spectroscopic Properties). The interatomic cysteiny1 sulfur atom distances fall into two classes, three with an average separation of 6.24 Å and three with an average separation of 6.55 Å. The combination of this trigonal distortion of the cysteines
(defined by the 3 axis), and the tetragonal compression (4 axis) of the cluster described before, result in a unique cluster orientation (Figure 2). The remaining symmetry element is reflective symmetry of an effective mirror plane passing through atoms Fe-1, S$_3^*$, Fe-2, and S$_4^*$ of the cluster. The polypeptide chain interacting with this presumably prochiral cluster induces an asymmetry that can be assigned the configuration S. As already mentioned, TYR-19 induces further asymmetry, creating a diastereomer described as S-S. In addition, the side chains of LEU-17, PHE-48, MET-49, LEU-65, PHE-66, ILE-71, TRP-76, TRP-80 all approach the cluster within five Å. It is suggested that the asymmetric interactions that these residues may have with the cluster would exert much smaller effects or reinforce the previously mentioned ones (Carter 1977b).

Cysteines 46, 63 and 77 accept NH $\cdots$ S hydrogen bonds from amides in hairpin turn geometries displaced N+2 in the sequence. Cysteine 46 also accepts an additional hydrogen bond from an amide elsewhere in the polypeptide (N-81). The two NH $\cdots$ S$^*$ hydrogen bonds in HiPIP involve the amide nitrogens N-49 and N-77. These six NH $\cdots$ S hydrogen bonds are arranged in pairs with each member of the pair being donated from opposite sides of the effective mirror plane of the cluster and thus reinforcing the reflective symmetry of the cluster (Carter 1977b).

A comparison of oxidized and reduced HiPIP structures has revealed, as mentioned above, that upon reduction the cluster expands 0.16Å (average of four bonds) along the directions of the Fe-1-S$_2^*$ direction and 0.08 Å (average of 4 bonds) along the Fe-1-S$_1^*$ direction.
The four Fe-S* bonds parallel to the mirror plane and the 4 axis contract by 0.03 Å (Figure 2); or in other words, an overall 0.06 Å expansion of the cluster occurs upon reduction. Since the Fe-S bond lengths determined by x-ray crystallographic methods at present refinement are accurate to ± 0.1 Å, it is important to note that the more accurate extended X-ray absorption fine structure technique (EXAFS) (± 0.02 Å) suggests that if any changes occur, a 0.01 Å contraction in the average Fe-S distance occurs with reduction (Teo and Shulman 1982). Thus, the oxidation-reduction dependent crystallographic cluster rearrangements should be viewed with some caution. Whichever results one wants to accept as providing the most representative picture of cluster redox structural changes, it appears clear that the changes are small, indicating low Franck-Condon barriers for oxidation and reduction.

Concomitant with these cluster rearrangements, changes in the polypeptide backbone occur with the NH ••• S* hydrogen bonds appearing to shorten and/or become more linear upon making the cluster more negative. The TYR-19 hydrogen bond network also changes upon reduction. The network consists of an NH (N-72) hydrogen bond to TYR-19 hydroxyl oxygen and donation of an O-H hydrogen bond from TYR-19 to a bound water on the protein surface. Upon reduction, TYR-19 moves closer to S3* and shortens the NH-72 O-TYR-19 hydrogen bond, indicating a possible electrostatic charge-dipole interaction between the more negatively charged cluster and TYR-19. In summary, these hydrogen bond networks are suggested to help delocalize the negative charge added to the cluster upon reduction (Carter et al. 1974a).
Kinetics

Although *C. vinosum* HiPIP has been kinetically studied rather extensively with both small reductants and oxidants (Mizrahi et al. 1976, Rawlings et al. 1976, Bennett 1977, Cummins and Gray 1977, Mizrahi et al. 1980, Feinberg and Johnson 1980, and Adzamli et al. 1981) and non-physiological redox proteins (Mizrahi and Cusanovich 1980, and Aprahamian and Feinberg 1981), the mechanism by which HiPIP undergoes electron transfer is still not known in detail.

Studies based primarily on the reactions of the iron hexacyanides with HiPIPs from *C. vinosum*, *T. pfennigii*, *Rr. gelatinosa* and *Paracoccus* sp. have been found to exhibit no kinetic complexity nor correlation with redox potentials (Mizrahi et al. 1980). Examining the effects of ionic strength on the kinetics of HiPIP oxidation by ferricyanide shows that electrostatic interactions between a particular HiPIP and ferricyanide seem to be affected by a localized charge and not by the total protein charge, as indicated by the similar ionic strength effects observed for three different negatively-charged HiPIPs. The fact that for all HiPIPs examined, the apparent charge on the reduced protein was greater than on the oxidized protein, independent of net protein charge, has been interpreted to mean that the charge of the cluster is observable by the reactant (Mizrahi et al. 1980). A possible model for the delocalization of the minus two net charge of the reduced cluster to the surface of the protein, where it could be seen by ferricyanide, has been presented. It involves the hydrogen bond network to the cluster inorganic and cysteiny1 sulfurs (see above), with coupled resonance with nearest neighbor polypeptide
carbonyls (Mizrahi et al. 1980). The model reconciles the observation that oxidized C. vinosum, and T. pfennigii HiPIPs demonstrate ionic strength independent ferrocyanide reduction kinetics by noting the existence of nearby positive charges which may result in an effective neutralization of the minus one charge of the oxidized cluster. Likewise, the plus-minus electrostatic interaction found for Rp. gelatinosa HiPIP with ferrocyanide was rationalized by the presence of several positive charges in the same region. It is of interest to note that the reduction of C. vinosum HiPIP by ascorbate and dithionite are also ionic strength independent (Mizrahi et al. 1976). Based on the proposed model, both oxidation and reduction would take place in a hydrophobic region where the cluster is closest to the protein surface.

Recently, in C. vinosum and Rp. gelatinosa HiPIPs, the sole HIS-42 has been suggested to also aid in delocalization of the cluster charge onto the surface of the protein (Nettesheim et al. 1983). The mechanism for this delocalization, especially in light of its relatively distant location from the cluster (10 Å) is unknown. The relationship and relative importance of HIS-42 to the postulated delocalization model discussed above remains to be seen. Further, the implications of such long distance delocalization in interpreting active site electrostatic interactions has not been addressed.

The study of the effect of pH on the kinetics of oxidation and reduction is often used to identify the involvement of mechanistically important amino acid side chains. Due to the instability of HiPIPs at pH values below 5 and above 11, only limited pH/rate constant profiles can be constructed. Nevertheless, it has been observed that Rp.
gelatinosa HiPIP/iron hexacyanide kinetics possess apparent pK values of 7.3 (reduction) and 7.8 (oxidation); and *C. vinosum* exhibits pH independent kinetics in the pH range of 7-11, but appears to have a pK below 6 (reduction) and below 7 (oxidation) (Mizrahi et al. 1980). Recently, the pH dependence of *C. vinosum* oxidation by ferricyanide has been reexamined (Feinberg and Johnson 1980). Using electrostatically corrected second order rate constants to account for changes in the net protein charge as a function of pH, they observed a marked pH dependence on the rate of ferricyanide oxidation in the region from pH 5-11. Specifically, two apparent pK's were observed: one at pH 7 and another at pH 10, with the former being assigned to HIS-42. A similar pK (7.1) is obtained for the pH-dependent oxidation (electrostatically uncorrected) of *C. vinosum* by 4,7-di(phenyl-4-sulfonate)-1,10-phenanthroline cobalt(III). It is of interest to note that this is the only reaction with any HiPIP examined so far for which saturation kinetics have been observed (Adzamli et al. 1981). The pK's estimated from the kinetics for *C. vinosum* and *Rp. gelatinosa* HiPIPs are in reasonable agreement with HIS-42 pK values determined by 1H-NMR (Nettesheim et al. 1983).

Other attempts to ascribe specific amino acid residues as having a role in the redox mechanism of these proteins include the suggestions that the invariant TYR-19 may mediate electron transfer to and from the cluster (Carter et al. 1974a). Although similar roles for tyrosine residues in other redox proteins have been suggested (Takano et al. 1973), none have stood the test of time. Evidence mitigating against this role, if one assumes that 8-Fe ferredoxins and HiPIPs
follow similar oxidation and reduction mechanisms, include chemical substitution of a leucine residue to replace one tyrosine residue of a 2[4Fe-4S] ferredoxin, without impairing its activity in a phosphoroclastic assay (Lode et al. 1974). In addition, direct electron transfer from phenols, and presumably protein bound tryosine side chains, to oxidized iron redox centers, has been suggested to be thermodynamically prohibited (Burns et al. 1976). Finally, reevaluation of the *C. vinosum* sequence (Tedro et al. 1981) invalidated the proposed role of ASP-45 (revised to ASN-45) in electron transfer (Mizrahi et al. 1976).

The mechanism for electron transport of such small hydrophilic redox reagents as Fe(EDTA)$^{2-}$ and Fe(CN)$_6^{3-}$ are processes dominated by entropic terms and small enthalpic contributions; while redox reagents such as Co(phen)$_3^{3+}$ and Ru(NH$_3$)$_5$py$_2^{3+}$ containing pi-conjugated ligands are dominated by enthalpic contributions. Based on the calculated, electrostatically corrected self-exchange rate constants, and the above activation parameters, it is suggested that reagents with aromatic groups are able to penetrate the hydrophobic region surrounding the cluster and more directly interact with the cluster. The presumably required movements of the polypeptide chain for penetration to occur, protein activation, would account for the large enthalpy term associated with these reactants (Rawlings et al. 1976, Cummins and Gray 1977). On the other hand, hydrophilic reagents like Fe(EDTA)$^{2-}$ cannot penetrate the protein and have to undergo electron transfer at a larger distance. A modified version of the Hopfield equation (Hopfield 1974) has been used to estimate the redox site to surface distance for
electron transfer between various reactants and HiPIP (Mauk et al. 1980). The distance calculated for Fe(EDTA)$^{2-}$ (5.8 Å) is larger than that for Ru(NH$_3$)$_5$Py$^{3+}$ (1.6 Å), consistent with the above accessibility arguments and with crystallographic estimates of the [4Fe-4S] center surface to protein surface contact distance (4-5 Å) (Mauk et al. 1980, Carter et al. 1974a).

The kinetics of oxidation and reduction reactions between HiPIPs and Q-type cytochromes are complex relative to the reactions with the small redox reagents. With certain cytochromes-Q$_2$, the reactions were biphasic, suggesting two possible orientations within the activated complex. Contrary to the iron hexacyanides, electrostatics play little role, and non-polar interactions appear to be predominant (Mizrahi and Cusanovich 1980, and Aprahamian and Feinberg 1980).
Rubredoxin

As a class, rubredoxins (Rds) are Fe-S protein that contain no labile sulfur and typically have a single iron atom nearly tetrahedrally coordinated to the sulfur of four cysteine residues (Figure 1) near the surface of the small protein. In 1965, the Rd from *Clostridium pasteurianum* was isolated in a highly purified form (Lovenberg and Sobel 1965). Since this time, Rd has been purified from many other anaerobic sources. These sources include five additional clostridial-type bacteria (Stadtman 1965, Lovenberg 1966, Mayhew and Peel 1966, Bachmayer et al. 1967, Yang et al. 1980); nine sulfate or sulfur reducing bacteria (Newman and Postgate 1968, Laishley et al. 1969, Bruschi and Le Gall 1971, Bruschi et al. 1977, Probst et al. 1978, Hatchikian et al. 1979, Moura et al. 1980, Le Gall et al. 1980); and five photosynthetic green sulfur bacteria (Meyer et al. 1971, Steinmetz and Fischer 1981, Steinmetz and Fisher 1982a,b).

Rds from aerobic sources have been reported (Kusunose et al. 1967, Peterson and Coon 1968, van Eyk and Bartels 1970). The aerobic Rd from *Pseudomonas oleovorans* (and perhaps other aerobic Rds) is atypical in that it contains either one or two Fe centers and is of a larger molecular weight (19 kilodaltons versus 6 kilodaltons for anaerobic Rds). Nevertheless, the principal physical and chemical properties of this Rd, including the sequence distribution of coordinating cysteine residues, are similar to the single Fe center Rds from anaerobic sources (Peterson and Coon 1968, Benson et al. 1971).
Structure

Anaerobic Rds consist of a single polypeptide chain 52 to 54 amino acids long. The amino acid sequences of five anaerobic Rds (Bachmayer et al. 1968a&b, Bruschi et al. 1976a&b) and a single aerobic Rd have been reported (McCarthy 1972). A characteristic of all Rds isolated to date is the preponderance of negative charges.

The three-dimensional structures of Rds from *C. pasteurianum* (Watenpaugh et al. 1980) and *Desulfovibrio vulgaris* (Adman et al. 1977) have been determined to 1.2 Å and 2.0 Å resolutions, respectively. In both proteins, the polypeptides are folded into a roughly three-sided box, with the sides of the box being formed by a minimally hydrogen bonded three-stranded antiparallel beta sheet, a zig-zag looped section and a random segment possessing a bend near the Fe center (Adman 1979).

The Fe center occupies one corner of the box and is coordinated by CYS 6, 9, 39, and 42. The interior of the protein contains hydrophobic residues, predominately closely-packed aromatic residues, which run down from the Fe center to the opposing side of the box. The Fe center is situated in a shallow hydrophobic cavity near the surface of the protein with at least one of the cysteiny1 sulfurs (CYS-42) probably solvent exposed. The only polar groups in the Fe center cavity, as with HiPIP, are NH \( \cdots \) S hydrogen bonds. Likewise, the hydrogen bonds are orientated so that the peptide-linked carbonyls are pointing outwards, suggesting their involvement in the partial neutralization of the negatively charged Fe center (Adman 1979).

The current crystallographic Fe-S bond distances for oxidized *C. pasteurianum* Rd agree well with the 2.30 Å average bond distance
determined by EXAFS measurements (Watenpaugh et al. 1980, Sayers, et al. 1976); nevertheless, earlier refinements suggested the existence of an anomalously short Fe–S bond (Watenpaugh et al. 1973). The Fe atom, to a first approximation, is tetrahedrally coordinated by four cysteines, with small distortions in Fe–S bond length and S–Fe–S bond angles present. Similar distortions from ideal tetrahedral symmetry are also observed in x-ray crystallographic analysis of isoelectronic Fe center analogs. Comparison of the structures of oxidized and reduced analogs show an average 0.09 Å expansion in Fe–S bond lengths (Lane et al. 1977). Technical difficulties in obtaining a refined structure of reduced Rd have prevented similar comparative measurements of the protein-bound center. Nevertheless, EXAFS studies of oxidized and reduced *Peptococcus aerogenes* Rd indicate that a similar increase in the average Fe–S bond lengths of 0.05 Å occurs upon reduction (Coucouvanis et al. 1976, Shulman et al. 1978).

Function

The specific biological function of Rd isolated from anaerobic sources is unknown. However, the 2-Fe center Rd from *P. oleovorans* is known to function as an electron carrier in an omega-hydroxylation system within this organism. As part of this pathway, Rd accepts electrons from a FAD-containing NADH:rubredoxin oxidoreductase (E.C. 1.6.99). A 1:1 complex between the Rd and its reductase is known to occur, although the catalytic importance of this complex has not been shown (cf. Lode and Coon 1973). As already mentioned, reduced rubredoxin acts as a specific electron donor to the hydroxylase of the
Anaerobic Rds can be rapidly reduced by this and other similar reductases; however, they are unable to support the enzymatic hydroxylation reaction (Lode and Coon 1973).

The NADH:rubredoxin reductases have been identified in a number of anaerobes, including Desulfovibrio gigas (Le Gall 1968), C. sticklandii, (Stadtman 1965) and C. thermoacetica (Yang and Ljungdahl 1977). In contrast to the rather broad specificity displayed by most reductases, the reductase from D. gigas is rather specific for the Rd isolated from that organism. Based on sequence differences between the Rds assayed, it was suggested that changes in the number and position of charged residues may account for the specificity (Le Gall et al. 1979). Many of these sequence substitutions occur in a highly charged region away from the Fe center. This region has been suggested to be of possible importance as a recognition site (Watenpaugh et al. 1979). A ring of conserved negative charges (residues 50, 48, 36, 19, and 14), in a closer proximity to the Fe center, has also been postulated as playing an important role in the interaction of Rd with the reductase (Adman 1979). Ionic strength effects on spectrophotometric and ultracentrifugal studies of complex formation between spinach FNR and Megasphaera elsdenii Rd, and on a catalytic assay involving these proteins, provide experimental evidence for a plausible plus-minus electrostatic interaction (Foust et al. 1969).

Spectroscopic Properties

The visible absorption spectrum of the red-colored oxidized Rd (lambda max = 570, 490, 380, and 350 nm) is the most structured of any
simple Fe-S protein. Upon one-electron reduction, Rd appears colorless ($\lambda_{\text{max}} \approx 333$ nm) with only a pale yellow color being observed at very high Rd concentrations. Like other simple Fe-S proteins, the visible spectrum arises from $S \rightarrow Fe$ charge transfer transitions (Eaton and Lovenberg 1973). In the oxidized state Rd gives a completely rhombic EPR signal ($g = 4.3$), which is attributable to the Fe(III) in a low symmetry environment. Reduced Rd is EPR silent. Mossbauer spectra have shown the iron in both oxidation states to be high spin. Due to the large magnetic moments, 5.85 and 5.05 Bohr magnetons, associated with oxidized and reduced Rd, respectively, the hyperfine-shifted resonances in the $^1H$-NMR spectrum are considerably broadened in both oxidation states (Phillips et al. 1970b).

Redox Properties

The redox potential of *C. pasteurianum* Rd is $-57$ mV at pH 7 (Lovenberg and Sobel 1965). As a class, Rds exhibit a rather narrow range of redox potentials, +20 to $-61$ mV, in contrast to the ranges displayed by other simple Fe-S proteins (Meyer et al. 1971, cf. Bertrand and Gayda 1982 and references cited therein). To date, the variation in Rd redox potential with changes in pH, ionic strength, or temperature has not been examined. The negative electrostatic environment of the center created by mercaptide coordination and adjacent negatively charged side-chains has been suggested to be largely responsible for the lower redox potential of Rd compared to the Fe(III)/Fe(II) couple, $+770$ mV (Eaton and Lovenberg 1973). The redox potentials of synthetic analogs are much more negative ($\sim 600-700$ mV).
than that of Rd, suggesting that protein environmental factors other than electrostatics are having a significant role in modifying Rd redox potential (Lane et al. 1975).

To date, only a single transient kinetic study, that of C. pasteurianum Rd, has been reported (Jacks et al. 1974). The lack of kinetic characterization probably is a consequence, at least in part, of the relatively low Rd yields and the autooxidizable nature of the reduced protein (Lovenberg and Sobel 1965). Reduction of Rd by Ru(NH$_3$)$_6^{2+}$, V(H$_2$O)$_6^{2+}$, and Cr(H$_2$O)$_6^{2+}$ indicate that Rd undergoes outer-sphere reduction with a high intrinsic reactivity. A physical interpretation of this high reactivity may involve the relative exposure of the Fe center; but of probable greater significance is low protein reorganizational energy associated with reduction (a $\Delta G^+_{\text{reorg.}} < 1.3$ kcal/mol has been estimated from EXAFS and resonance Raman data; Reynolds et al. 1980). The Ru(NH$_3$)$_6^{2+}$ reduction of Rd was found to be essentially independent of pH (electrostatically uncorrected) in the pH range 6.3–8.0. Ionic strength effects on the V(H$_2$O)$_6^{2+}$ reaction at pH 4 are suggestive of a direct interaction of the reductant with the single negatively-charged oxidized Fe center (Jacks et al. 1974).
Flavin coenzymes are uniquely capable of participating in either one or two-electron transfer reactions. Although flavins, through their three possible oxidation states, can participate in a variety of interesting chemical reactions, the following discussion will be limited to topics most closely related to flavosemiquinone reactivity (for reviews see Edmondson and Tollin 1983, Muller 1983).

Free Flavin Redox and Protonation States

Available redox states of free flavins, their visible region spectroscopic properties and relevant protonation states are summarized in Figure 4. Characterization of free flavosemiquinones is hindered by a rapid disproportionation reaction which results in the formation of a pH-dependent equilibrium mixture of the flavoquinone ($F_{\text{ox}}$), flavosemiquinone ($F_{\text{H}^\cdot}$), and flavohydroquinone ($F_{\text{red}H_2}$) forms, equation (1).

$$2 F_{\text{H}^\cdot} \rightleftharpoons F_{\text{ox}} + F_{\text{red}H_2} \quad (1)$$

Thus characterization of free flavosemiquinone properties and reactivity has largely relied on the rapid measurement techniques of laser flash photolysis and pulse radiolysis, or the use of solution conditions and/or structurally modified flavins that stabilize flavosemiquinones (cf. Edmondson and Tollin 1983 and references cited therein).
Figure 4. Spectral, redox, and protonation states of riboflavin.

R = ribityl, Fl_{ox}H = flavoquinone, FlH_{2} = neutral flavosemiquinone, Fl_{red}H_{3} = flavohydroquinone. Numbers below each flavin species is their visible absorption maxima (millimolar extinction coefficient).
Each redox state of flavin is amphoteric (Figure 4). Additional ionizable groups may be introduced through exocyclic substitution. The absorption spectrum of (A) has visible maxima at approximately 450 and 375 nm, which may be altered significantly upon ring substitution or solvents (for a review see Penzer and Radda 1971). Ionization of the N-3 position of (A) shifts the lower wavelength band hypsochromically and hyperchromically without much alteration in the long wavelength band (Figure 4); and it also results in the OH⁻ quenching of the fluorescence of (A). The pK for the N-5 deprotonation of flavosemiquinone (approximately 8.4 for free riboflavin) has been determined by EPR (Ehrenberg et al. 1967), potentiometry (Draper and Ingraham 1968), pulse radiolysis (Land and Swallow 1969), and laser flash photolysis (Vaish and Tollin 1971). Major visible region spectral differences (Land and Swallow 1969) exist between neutral (C) and anionic (D) flavosemiquinones (Figure 4). Additional differences in EPR properties and reactivity occur between the two forms (see Free Flavin: Flavosemiquinone EPR and Flavosemiquinone Oxidation). The negative charge on (D) can be placed on all hetero atoms of the pyrimidine ring except N-3 (cf. Muller et al. 1970). The weakly colored flavohydroquinone (1,5 dihydroflavin, other isomeric forms can exist) undergoes ionization at N-1 (E) with the negative charge distributed as in (D) (Figure 4, cf. Gishla et al. 1974 and references cited therein).

Substitution of the flavin ring results in substantial alterations in the pK's for the above ionizations. For neutral flavosemiquinone deprotonation, the pK varies in the following order:
8-alpha-substituted flavins < riboflavin = lumiflavin < FMN (Vaish and Tollin 1971, Edmondson et al. 1977). Appendix A summarizes the structures, abbreviations, and redox potentials of the flavin analogs to be discussed later.

Potentiometric titrations of riboflavin and FMN give $E_{m,7}$ values for the oxidized semiquinone redox transition (reaction 1 in Figure 4) of -231 and -238 mV, respectively. For the semiquinone/fully-reduced redox couple (reaction 2) the values are -167 and -172 mV, respectively. For riboflavin, the oxidized/semiquinone is -146 mV at pH 5 and -325 mV at pH 10 (Ingraham and Draper 1968). For 5-deazariboflavin, the oxidized to semiquinone redox couple is estimated to be -650 mV, making it a useful reductant for low potential redox proteins (Blakenhorn 1976, Massey and Hemmerich 1978). For other analogs only the two-electron redox potential values have been reported (Appendix A).

Flavosemiquinone EPR

Utilization of N-5-alkylated flavins (Muller et al. 1970) and flavins in organic solvents (Erhenberg et al. 1967) has allowed EPR studies of the neutral and anionic semiquinones, respectively. These studies have demonstrated that the majority of unpaired spin density resides in the C-4a, N-5, C-8, and N-10 positions, in both the neutral and anionic semiquinone forms. Nevertheless, significant differences in spin density distributions between the two forms occur at positions C-4a, C-6, C-8, and C-9. Such results suggest that these positions may be important as sites for electron transfer and may also be involved in
determining the observed differences in the reactivity between neutral and anionic flavosemiquinones (see Flavosemiquinone Oxidation).

Flavosemiquinone Oxidation

As yet, only limited evidence has been presented for a specific site of electron transfer for free flavosemiquinones. Most studies have examined oxidation of flavosemiquinone rapidly generated by flash photolysis (Vaish and Tollin 1971, Tollin and Rizzuto 1975, Edmondson et al. 1977). The process of flavin photoreduction by EDTA, a commonly used photoreductant, is outlined in Figure 5. Addition of oxidants to the flavin/EDTA system allows the rate constants for electron transfer from flavosemiquinone and, under certain conditions, flavohydroquinone, to be monitored by changes in absorbance (Figure 5). Neutral flavosemiquinones disproportionate faster than anionic species (Vaish and Tollin 1971). Flavins with charged substituents exhibit an ionic strength effect on the disproportionation rate constant (Vaish and Tollin 1971). Although a similar effect would be expected for unsubstituted anionic flavosemiquinones, this has not been examined. Of interest is the fact that different 8-alpha-substitutions produce only minor changes in the rate of disproportionation, suggesting this position is not important in electron transfer between free flavosemiquinones (Edmondson et al. 1977).

Much of the early work on free flavosemiquinone oxidation used small molecule oxidants and examined the differences in reactivity between neutral and anionic flavosemiquinones as a function of pH. These experiments have shown at least a $10^4$ fold difference in
reactivity between neutral and anionic flavin radicals for dioxygen oxidation; while ferricyanide oxidation shows minimal pH effects (Vaish and Tollin 1971). Since the N-5 position is the site of deprotonation in neutral flavosemiquinones, the interpretation presented was that dioxygen and similar strongly pH-dependent oxidants react at or very near the N-5 position; while ferricyanide reacts at a site removed from the N-5 position, e.g. the dimethylbenzene ring, perhaps at C-8 where the majority of the spin density in the dimethylbenzene ring resides (Vaish and Tollin 1971, Tollin and Rizzuto 1975).

Little variation is observed in the rate constants for the small oxidant reactions with different free flavin analogs. A notable exception is the ferricyanide reaction, for which Lf and Rf yield identical rate constants, while the rate constants for 10-MI and 7,8-diClRf are 2-3 fold lower (Tollin and Rizzuto 1975). Although, as noted by the authors, the substitutions between these groups of analogs occur at the 7 and 8 positions of the dimethylbenzene ring, the differences in rate constants are probably not simply due to structural effects, but are more likely due to a combination of factors including redox potential and alterations in the flavosemiquinone pK. Indeed, the lower rate constant for dioxygen oxidation of anionic 8-alpha-substituted flavosemiquinones, as compared to Rf, has been attributed to the generally higher redox potentials of 8-alpha-substituted flavins (Edmondson et al. 1977).

Other work has examined the oxidation of various C-7, C-8, C-8-alpha, and N-10 substituted analogs by horse heart cyochrome c (Ahmad et al. 1981). Based on the differences in magnitude of the ionic
strength effects seen between 8-alpha and N-10 charge-bearing flavins, and of changes in rate constant seen with different sized substituents at C-7 and C-8, it was concluded that electron transfer involves the N-5-dimethylbenzene region of the flavin moiety. Factors other than structural or electrostatic factors, e.g. redox potential or unpaired electron density distribution, were found to give no correlation with the observed rate constants.
Figure 5. Process of free flavin photoreduction by EDTA and subsequent oxidation of reduced flavin forms by exogenous oxidant.

$F_{ox}H = $ flavoquinone, $\textbf{3}_{ox}H^* = $ excited triplet state of flavoquinone, $F_{H} = $ neutral flavosemiquinone, $F_{red}H_2^* = $ anionic flavohydroquinone, $X_{ox}$ and $X_{red} = $ oxidized and reduced state of exogenous oxidant, respectively, EDTA = ethylenediaminetetraacetic acid.
Flavodoxin

Flavodoxin is an FMN-containing flavoprotein that functions as a low potential one-electron mediator in many microbial metabolic pathways. The name flavodoxin is derived from its ability to functionally substitute for $2[4\text{Fe}-4\text{S}]^{2+},^{1+}$ ferredoxins in many ferredoxin-dependent reactions. Indeed, in many bacteria, the expression of flavodoxin is induced and ferredoxin synthesis suppressed under low-iron growth conditions (Knight and Hardy 1966). The structural, physical, and chemical properties of flavodoxin have been extensively reviewed (Mayhew and Ludwig 1975, Simondsen and Tollin 1980); and this discussion will attempt only to highlight flavodoxin redox properties.

Redox States and FMN Environment

Upon one-electron reduction of the single FMN of the yellow, oxidized Clostridium MP flavodoxin ($\lambda_{\text{max}} \approx 445$ and 376 nm), a blue-colored neutral flavosemiquinone results (a broad maximum with structure at 617, 575, 505, 376, and 350 nm). Thermodynamic stabilization of the neutral flavosemiquinone results from protein hydrogen bond interactions with the N-5 position of FMN shifting its $pK_a$ to a higher value than in free FMN (cf. Mayhew and Ludwig 1975 and Simondsen and Tollin 1980 and references cited therein). Disproportionation of the flavodoxin semiquinone is extremely slow, and
thus has a negligible contribution to any of the relatively rapid reactions studied. Addition of a second electron produces a weakly yellow-colored (\(\lambda_{\text{max}} \approx 450\) and 365 nm) anionic fully-reduced flavodoxin (cf. Mayhew and Ludwig 1975 and references cited therein).

X-ray crystallographic studies have been performed on all three oxidation levels of *Clostridium* MP flavodoxin. The FMN prosthetic group is planar in all three oxidation states, although the flavohydroquinone state does deviate slightly from a completely planar structure. Restriction of the anionic flavohydroquinone to a nearly planar structure by specific polypeptide interactions with FMN is suggested to account for the large differences between the flavoquinone/flavosemiquinone (-92 mV) and the flavosemiquinone/flavohydroquinone (-399 mV) redox couples for *Clostridium* MP flavodoxin (reviewed in Mayhew and Ludwig 1975, Simonsen and Tollin 1980). This contrasts with the relatively small difference in potentials for the same couples observed with free FMN (see above).

In both *Clostridium* MP and *Desulfovibrio vulgaris* flavodoxins, X-ray crystallographic analysis have shown the FMN moiety to be located near the surface of these low molecular weight, highly-structured proteins. The flavin ring system is sandwiched by large hydrophobic side chains with the buried pyrimidine ring being highly hydrogen bonded. The dimethylbenzene ring of FMN is the only portion of the flavin with significant solvent accessibility (Mayhew and Ludwig 1975). This fact, coupled with EPR and ENDOR results which suggest, that as in free flavins, a substantial amount of unpaired electron spin density occurs at position C-8 (Palmer et al. 1971, Eriksson and Ehrenberg
1973), have prompted the suggestion of electron transfer occurring through the dimethylbenzene ring (Mayhew and Ludwig 1975, Jung and Tollin 1981, Simonsen and Tollin 1983). Kinetic and computer modeling studies of flavodoxin have provided experimental support for the involvement of the dimethylbenzene ring in electron transfer from flavodoxin semiquinone (see Flavodoxin: Kinetics).

Kinetics

The majority of flavodoxin kinetic studies have used the Cyanoacetate protein which is structurally homologous to that from Clostridium MP flavodoxin, especially in the region of the flavin binding site (Dubourdieu and Fox 1977). Although quantitative differences in semiquinone reactivity with various small oxidants exist among flavodoxins, the general trends are the same and therefore only the kinetic results with Cyanoacetate, the source used in the dissertation studies, will be discussed.

Flavodoxin reactivity with small oxidants such as ferricyanide and dioxygen are much slower than seen with free flavins. The lower flavodoxin dioxygen reactivity vs. free FMN (3.2 M⁻¹s⁻¹ vs. 1.4 x 10⁷ M⁻¹s⁻¹) has been ascribed to the higher pK for FMN bound to flavodoxin (reducing the proportion of the much more reactive anionic flavosemiquinone), and to the buried nature of the N-5 position, the presumed region of dioxygen electron transfer with free flavin (Tollin and Rizzuto 1975). The pH dependence of the oxygen reaction of flavodoxin has not been reported, but the faster rate of oxidation with diClFMN-substituted flavodoxin and riboflavin-substituted flavodoxin
support the importance of semiquinone pK shifts in dioxygen reactivity with flavodoxin (Shiga and Tollin 1976). Free diC1Rf, as a result of electronic effects, is suggested to have a lower pK for the flavosemiquinone (Rizzuto and Tollin unpublished results cited in Tollin and Rizzuto 1975) and riboflavin substitution presumably causes a protein conformational change that lowers the protein-bound flavin semiquinone pK. Flavodoxin semiquinone reactivity with ferricyanide, which with free flavins (see above) was suggested to react through the dimethylbenzene ring, is faster than the flavodoxin dioxygen reaction which was suggested to react near the N-5 position (160 M⁻¹s⁻¹ vs. 3.2 M⁻¹s⁻¹, Shiga and Tollin 1976). The much lower rate constant for flavodoxin semiquinone oxidation by ferricyanide, compared to the essentially pH-independent reaction of free flavins with ferricyanide (∼6 x 10⁸ M⁻¹s⁻¹; Vaish and Tollin 1971), is suggested to arise from protein effects other than pK shifts, such as flavin-tryptophan interactions (Gillard and Tollin 1974). The diC1FMN flavodoxin reacts more slowly with ferricyanide than native flavodoxin. In addition, the diC1FMN flavodoxin shows saturation effects that are apparently absent with native flavodoxin (Jung and Tollin 1981). The reaction of native flavodoxin and ferricyanide has been found to be strongly ionic strength-dependent, with minus-minus electrostatic interactions being indicated. These results were found to be consistent with structural properties of putative encounter complexes between ferricyanide and flavodoxin generated by computer graphics. In the complex, ferricyanide is in van der Waals radii contact distance of the dimethylbenzene ring of the flavin (Simonsen et al. 1982).
Oxidation of native flavodoxin with horse heart cytochrome c, like ferricyanide, is strongly ionic strength-dependent; however, in this case, the expected plus-minus interaction is indicated. In contrast to the ferricyanide reaction, native flavodoxin (as well as diClFMN flavodoxin) exhibits saturation behavior. Both the second order and first order rate constants are ionic strength dependent, increasing with decreasing ionic strength. The ionic strength dependence of the first order process is suggested to represent an electrostatic-dependent conformational change within the collisional complex. In addition, the observed first order rate constant has a contribution from the electron transfer since the plots of logarithm of the rate (constant) plotted as a function of the redox potential difference between the chlorine-substituted FMN analogs of flavodoxin (see below), and various oxidants, are in agreement with the potential dependence of modified outer-sphere electron transfer relationships (Jung and Tollin 1981, Simonsen and Tollin 1983). Computer modeling studies of a putative complex between cytochrome c and flavodoxin again give results which are consistent with the observed kinetics and suggest the involvement of the dimethylbenzene ring in electron transfer (Simonsen et al. 1982). Semiquinone oxidation kinetic studies of flavodoxin substituted with the analogs 7-ClFMN, 8-ClFMN, 7,8-diClFMN, and 5,6,7,8-tetrahydroFMN by cytochrome c have been interpreted in terms of electrostatic, electronic, and structural variations in the flavin analogs (Simonsen and Tollin 1983).
Ferredoxin:NADP+–Reductase

The chloroplast FAD-containing flavoprotein, ferredoxin:NADP+–reductase (E.C. 1.18.1.2) or FNR, catalyses the reduction of NADP+ to NADPH (Equation 2), which serves as the primary reductant in many metabolic pathways within the plant (Shin et al. 1963). Although the enzyme has been isolated from a number of sources, spinach FNR remains the most extensively characterized (see Zanetti and Curti 1980), presumably a result of a readily available supply of starting material.

$$2 \text{Fd}_{\text{red}} + \text{NADP}^+ + \text{H}^+ \xrightarrow{\text{FNR}} 2 \text{Fd}_{\text{ox}} + \text{NADPH} \quad (2)$$

Properties

Purified spinach FNR demonstrates up to eight isoelectric forms, of which the predominant one has a pH of 5.9 and a molecular weight of 32.5 kilodaltons. All isoelectric forms exhibit catalytic activity in NADPH-diaphorase assays, strong binding to ferredoxin-Sepharose 4B affinity columns, and spectral properties of the unseparated flavoprotein mixture. Quantitative differences between that of isolated FNR forms have not been reported. The molecular heterogeneity presumably results from a contaminating spinach leaf protease (Hasumi et al. 1983). The slow one-electron reduction of FNR using either dithionite or EDTA-light, produces the neutral semiquinone (Massey et al. 1970). The oxidized/semiquinone redox potential of FNR is -320 mV at pH 7 (Keirns and Wang 1972; however see also Batie and
Kamin 1981; Smith et al. 1981). X-ray studies of the three dimensional structure of spinach FNR are still at the preliminary stages (Karplus and Herriott 1982). Through the substitution of flavin analogs into FNR, it has been suggested, that, like flavodoxins, the C-8 position of the flavin is exposed to solvent, while the pyrimidine ring is buried within the protein (Zanetti et al. 1983).

Spinach FNR has been found to form complexes with many different types of redox proteins (Foust et al. 1969). The apparently non-specific nature of these interactions may arise from the limited requirement of a high, net negatively-charged redox protein. Complexation has been identified by spectrophotometric, ultracentrifugal, and column chromatographic methods (Foust et al. 1969, and Shin 1973). The complexes are rapidly formed \( > 10^8 \text{ M}^{-1}\text{s}^{-1} \) (Foust et al. 1969) and are influenced by ionic strength, pH, and the redox state of the proteins (Foust et al. 1969, Batie and Kamin 1981). Complexation between the physiological redox partners, spinach [2Fe-2S] Fd and FNR, results in a drop of the Fd potential by 22-92 mV; while the FNR potential is increased by \( \leq 23 \text{ mV} \) (Smith et al. 1981, Batie and Kamin 1981).

Little is known about the electron transfer rates between the components of Equation 2 (in the direction to the right), due to the rapid nature of these reactions. A recent pulse radiolysis study, however, has initiated some efforts to measure electron transfer kinetics of the components in the reaction to the left. The results have suggested that reduction of FNR to the semiquinone oxidation level by radiation-generated NADP free radicals occurs via a fast, ionic
strength dependent intramolecular electron transfer ($2.3 \times 10^4 \text{ s}^{-1}$ at $I = 0.005 \text{ M}$). In the absence of additional reactants, the FNR semiquinone is suggested to slowly disproportionate. Upon addition of oxidized spinach Fd, a first order process is observed with a rate constant of $2.36 \text{ s}^{-1}$. This reaction has been interpreted to represent intracomplex electron transfer from the FNR semiquinone to oxidized Fd (Maskiewicz and Bielski 1982).
**Proposed Research**

The goal of this research is to identify and quantitate factors which influence flavosemiquinone kinetic reactivity with Fe-S centers. Two transient kinetic techniques will be utilized in the experiments, laser flash photolysis and stopped-flow spectrophotometry. Laser flash photolysis of free flavins can rapidly generate free flavosemiquinones and from these, protein-bound flavosemiquinones in situ under anaerobic conditions. The technique also provides the capability to follow reactions whose rate constants are outside the time resolution of mixing methods. On the other hand, stopped-flow techniques allows the measurement, over a wider wavelength range, of much slower reactions involving stable flavosemiquinones, with a reduced number of potentially complicating reactions. The particular experimental systems to be used and the type of information hoped to be gained are discussed below.

1. The oxidation of structurally well-defined free flavosemiquinones (produced by laser flash photolysis) by a variety of oxidants under different solution conditions (pH and ionic strength) will be examined.

Rate constant-pH profiles for the oxidation of different flavosemiquinones by *C. vinosum* HiPIP (which exhibits only a small pH dependence for ferrocyanide reduction at pH values below 7 and is the most extensively characterized HiPIP example) will be measured to examine the reactivity of the neutral versus anionic flavosemiquinone
forms. The observed rate constant-pH data will be analysed to determine the intrinsic rate constants for oxidation of the neutral versus anionic flavosemiquinone forms. Any differences observed between the two forms will be related to the already elucidated differences in chemical and physical properties between the two species. For comparison, the pH dependence of flavosemiquinone oxidation by several well characterized inorganic oxidants will be made.

The rate constants for flavosemiquinone (lumiflavin) oxidation at a single pH and/or for a single flavosemiquinone form by both HiPIPs and inorganic oxidants with different redox potentials will be examined for correlations with the redox potential difference between the reactants (ΔE). Quantitation of the results will be attempted using the semi-empirical Marcus (1968) exponential equation which relates the dependence of the second order rate constants on ΔE. The possible introduction of steric, electronic, or solvation effects resulting from various specific flavosemiquinone exocyclic substitutions will also be examined.

The combined effects of electrostatics and redox potential (and possibly steric) will be examined by measuring the ionic strength dependence of FMN semiquinone oxidation by various HiPIPs of different redox potentials. The ionic strength-rate constant data will be electrostatically corrected (to infinite ionic strength) using the parallel plate bimolecular electrostatic equation of Watkins (Watkins 1984). The rate constant at infinite ionic strength (k∞) will be analysed by the Marcus equation, as well as the Hopfield treatment for vibronically assisted electron tunneling (Hopfield 1974), to determine
if, as above, $\Delta E$ has an effect on FMN semiquinone oxidation.

2. Stopped-flow studies of *C. pasteurianum* flavodoxin semiquinone oxidation by the same HiPIPs used in the FMN study will be measured at different ionic strengths. The ionic strength-rate constant data will again be electrostatically corrected (to infinite ionic strength) with $k_\infty$ being analysed for a dependence on $\Delta E$ in terms of the Marcus and Hopfield equations. A comparison of FMN and flavodoxin results should indicate what effect placing a flavosemiquinone within a protein matrix has on factors influencing flavosemiquinone reactivity with at least one type of Fe-S protein and flavoprotein. In addition, information on the electrostatic interaction domain on HiPIP for reactions with flavodoxin (as well as FMN, see above) will be obtained. This information could provide support for a site on HiPIP involved in the electron transfer reactions from flavodoxin and FMN semiquinones.

3. Laser flash photolysis studies of *C. pasteurianum* Rd and spinach FNR as a function of different molar ratios of the two proteins, free flavin used as the reductant, and ionic strength will be measured. The large redox potential difference between the two proteins will allow the reactivity of lumiflavin semiquinone with the Fe center of Rd in a 1:1 complex with FNR (obtained from a 1:1 mixture of the proteins at low ionic strength) to be selectively measured and compared with the reactivity of free Rd. Examination of the kinetics of the 1:1 complex using a free flavosemiquinone which can reduce both FNR and Rd (5-deazariboflavin), should allow any intracomplex electron
transfer from FNR semiquinone to oxidized Rd to be observed. Such a reaction has no bimolecular component and thus the observed first order rate constant would presumably be influenced by factors involved in the electron transfer process itself. By measuring the 5-deazariboflavin reaction at excess FNR over Rd at low and high ionic strengths, it should be possible to compare the reactivity of free and bound Rd with free FNR semiquinone.

4. A CD survey of the two different native HiPIP oxidation states of available sources over the far UV to visible wavelength region could provide useful comparative protein conformational information for interpreting the above kinetic results. The measurement of redox potentials of previously uncharacterized HiPIPs will be performed. This information will be required for work proposed in 1 and 2 above. In addition, the above information would test Carter's prediction concerning a correlation between CD and HiPIP redox behavior.
EXPERIMENTAL PROCEDURES

Materials

Proteins

HiPIPs from the various sources were purified following the general procedure described by Bartsch (1978). All HiPIPs, except that from *C. vinosum*, were kindly provided by Dr. T. E. Meyer (University of Arizona, Tucson). Purity indices for the oxidized proteins and extinction coefficients used to calculate HiPIP concentrations are summarized in Appendix B. For those HiPIPs for which previously published extinction coefficients did not exist the molar $\Delta \varepsilon_{500\text{nm}}$ were estimated by one of two methods. The $\Delta \varepsilon_{500\text{nm}}$ for HiPIPs reducible by ferrocyanide, were calculated using the $E_m$ values for the HiPIPs determined by the spectro-electrochemical measurements (see below) and the absorbance changes observed in the method of mixtures experiment (see below) in a form of the Nernst equation relating the HiPIP couple to the ferricyanide/ferrocyanide couple (cf. Lovenberg and Sobel 1965; Wilson 1978). For those HiPIPs not appreciably reduced by ferrocyanide (HiPIPs from the *Ectothiorhodospira* sps.), the $\Delta \varepsilon_{500\text{nm}}$ were estimated using an average $E_{375\text{nm},\text{ox}}$ (19.4 $\times$ 10$^3$ M$^{-1}$ cm$^{-1}$) for *Chromatium vinosum* and *Rhodopseudomonas gelatinosa* HiPIPs (Dus et al. 1967) and the spectral properties of the oxidized and reduced oxidation states of the given protein.
**C. pasteurianum** (ATCC 6013) cell paste was a gift from Dr. R. Simonsen. The Rd from **C. pasteurianum** was isolated and partially purified by the procedure in Armstrong *et al.* (1980) with final purification ($A_{280}^{ox}/A_{490}^{ox} = 2.4$) using ammonium sulfate chromatography as described by Lovenberg (1972). Rd$^{ox}$ concentrations were determined using an extinction coefficient at 530 nm of $3.7 \times 10^3$ M$^{-1}$ cm$^{-1}$. This value was estimated from the average 490 nm molar extinction coefficient of ten Rd$^{s}$ ($6.3 \times 10^3$ M$^{-1}$ cm$^{-1}$) and the relative absorbances at 490 nm and 530 nm of oxidized **C. pasteurianum** Rd (Lovenberg and Sobel 1965, Atherton *et al.* 1966, Newman and Postgate 1968, Bachmayer *et al.* 1968, Bruschi *et al.* 1977, Moura *et al.* 1977, Probst *et al.* 1978, Moura *et al.* 1980, Yang *et al.* 1980).

Spinach FNR was purified ($A_{458}^{ox}/A_{275}^{ox} = 0.12$) according to the method of Borchert and Wessels (1970), although a few experiments were performed with FNR that was a gift from Dr. E. Gross. An extinction coefficient of $10.3 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 458 nm was used to determine oxidized FNR concentrations (Foust *et al.* 1969).

**C. pasteurianum** flavodoxin was obtained according to published procedures (Mayhew 1971) with oxidized flavodoxin concentrations determined using a 445 nm molar extinction of $10.4 \times 10^3$ M$^{-1}$ cm$^{-1}$.

Tuna heart cytochrome c (type XI) was obtained from Sigma Chemical Company and was used without further purification. An extinction coefficient at 550 nm of $30 \times 10^3$ M$^{-1}$ cm$^{-1}$ for the reduced cytochrome was used to measure concentrations. Oxidized stocks were corrected for the percentage of reduced cytochrome present ($<15\%$).
Reagents

Potassium ferricyanide was purchased from Fisher Scientific Company and a $\varepsilon_{418} = 1.14 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used to estimate concentrations (Szentrimay et al. 1977). 1,1'-bis(hydroxymethyl)ferrocene was from Polysciences Inc. The oxidized species, ferricinium ion, was generated coulometrically with concentrations determined using an $\varepsilon_{638}^{\text{ox}} = 385 \text{ M}^{-1} \text{ cm}^{-1}$ (Szentrimay et al. 1977). Tris-(1:10-phenanthroline)cobalt(III)perchlorate dihydrate (Co(phen)$_3^{3+}$) was prepared as in Shilt and Taylor (1959) and was a gift of Mr. Glen Cheddar. An $\varepsilon_{350} = 3.7 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$ was used to estimate concentrations (Przystas and Sutin 1973). Fe(III)EDTA was prepared as previously described (Simonsen et al. 1982). All non-biological oxidant stock solutions were prepared immediately prior to use. The extinction coefficients of all reactants were assumed to be constant under all experimental solution conditions. Methylviologen was obtained from Sigma Chemical Company, indigotetrasulfonate from K & K Laboratories, and sodium dithionite from Vine Chemicals Limited.

Riboflavin was purchased from Sigma Chemical Company. Lumiflavin and 10-methylisoalloxazine were prepared as previously described (Guzzo and Tollin 1964). 7,8-dichlororiboflavin was synthesized as described (Shiga and Tollin 1976). 7-chlororiboflavin and 8-chlororiboflavin were gifts from Dr. J. P. Lambooy. The 8-alpha-(S-mercaptopropionic acid) riboflavin was a gift from Dr. D. B. McCormick. Oxidation of the latter analog to the sulphone level followed the procedure of Walker et al. 1971. The oxidation product was purified by thin-layer chromatography under dim light (silica gel H
type 60, 4:1:1 n-butanol:acetic acid:water, V/V/V) extracted with doubly distilled water, and lyophilized. FMN was obtained from Sigma Chemical Company and was purified (Nagel et al. 1982), under dim light, by passage through a Biogel P-2 column equilibrated with doubly distilled H₂O and lyophilized. Structures and redox potentials of the analogs are given in Appendix A. All other chemicals were of reagent grade from J. T. Baker Chemical Company, MCB Manufacturing Chemists Inc., or Sigma Chemical Company.
**Methods**

**Kinetics**

Oxidation of HiP IPs was performed with potassium ferricyanide with excess oxidant removed either by passage through a Sephadex G-25 (coarse) column equilibrated 1 mM potassium phosphate, pH 7 buffer and/or by several exchanges with the same buffer using an Amicon Model 12 pressure dialysis cell and a YM-5 membrane. Concentrations of oxidized HiPIP stock solutions were corrected for the amount of reduced HiPIP present (< 20%).

*C. pasteurianum* flavodoxin/HiPIP experiments were performed as a combined effort with Mr. Glen Cheddar of Dr. G. Tollin's laboratory (University of Arizona, Tucson). The stopped-flow apparatus used in these reactions has been described previously (Jung and Tollin 1981) as has the experimental procedure for the preparation of flavodoxin semiquinone and anaerobic solutions of the reactants (Simonsen et al. 1982). The stopped-flow experiments were carried out in 5 mM potassium phosphate, pH 7.2. The temperature was thermostated at 23.5°C. Ionic strength was varied by the addition of appropriate amounts of NaCl. Pseudo first order conditions were employed in which the concentration of oxidized HiPIP was in large excess over that for flavodoxin semiquinone. The *in situ* photoproduction of flavodoxin semiquinone by the 5-deazariboflavin/EDTA system (Simonsen et al. 1982) also generates fully-reduced flavodoxin. Although the exact amount of
fully-reduced flavodoxin produced is unknown, the small decrease in oxidized HiPIP concentration resulting from these additional reducing equivalents was corrected for by subtracting the amount of oxidized flavodoxin used (i.e. assuming all flavodoxin gets converted to fully-reduced form). Reactions were observed at 580 nm, a wavelength which allows the disappearance of the flavodoxin semiquinone to be followed.

The laser flash apparatus has been previously described (Simonsen and Tollin 1983) as well as the procedure for degassing samples (Cusanovich and Tollin 1980). All experiments were carried out at ambient temperature (20-25°C). For HiPIP and cytochrome c reactions, the flavin concentrations were typically 40 micromolar for all analogs, except the 8-alpha-(S-mercaptopropionic acid) riboflavin and its sulphone derivative for which 100 micromolar solutions were used. For reactions using 5-deazariboflavin 70 micromolar solutions were used. Luminflavin and 5-deazariboflavin reactions with the various HiPIP sources were carried out in 20 mM potassium phosphate, 10 mM EDTA, pH 7 buffer. All flavin solutions were freshly prepared using doubly distilled water. Buffers used in the redox protein/flavin pH studies contained 10 mM EDTA as the photosubstrate and the appropriate amounts of sodium phosphate or sodium pyrophosphate to give a final ionic strength of 0.24 M. Solutions for the simple oxidant reactions contained 5 mM L-methionine as the photosubstrate in sodium formate (pH 3 and 5) or sodium phosphate (pH 7.8) buffers, the final ionic strength (0.26 M) being adjusted with KCl. The dilution method was utilized for the pH studies, with redox protein additions made from concentrated stock solutions in 1 mM potassium phosphate, pH 7, and simple oxidants
from stocks in 0.26 M KCl, except for Fe(III)EDTA which was in pH 5 buffer. The pH was measured after the laser flash experiments and this pH value was used for subsequent data analysis. All pH measurements were made using a Sensorex S-200C combination electrode and an Instrumentation Laboratory Inc. model 205 digital meter. Buffers for the HiPIP/FMN semiquinone reactions contained 10 mM potassium phosphate, 5 mM EDTA and appropriate amounts of KCl to give the indicated ionic strength. FMN solutions were prepared immediately prior to use and contained approximately 40 micromolar FMN.

Data for N-5 containing flavins were obtained at 565 nm for HiPIP and cytochrome c reactions and at 545 nm for non-biological oxidant reactions. Kinetic transients with 5-deazariboflavin were obtained at 500 nm.

*C. pasteurianum* Rd/spinach FNR laser flash experiments were a collaborative effort with Mr. Anjan Bhattacharyya of Dr. Tollin's laboratory (University of Arizona, Tucson). The reactions were carried out in 4 mM potassium phosphate, 0.5 mM EDTA pH 7 buffer. Flavin concentrations were typically 90 micromolar for 5-deazariboflavin and 50 micromolar for lumiflavin. Kinetic transients obtained with 5-deazariboflavin were monitored at the indicated wavelengths and lumiflavin reactions were monitored at 540 nm. Ionic strength was varied by addition of the appropriate amount of KCl.

**Spectroscopy**

All near UV and visible electronic absorption spectra were taken with a Cary Model 118 recording spectrophotometer at ambient
temperature. CD spectra were measured at ambient temperature (22-25°C) or thermostated at 25°C using a water-jacketed 10 mm pathlength cell. All measurements were made on a Cary 60 with a 6001 CD attachment.

HiPIP samples for CD measurements were exchanged into 10 mM Tris-cacodylate, pH 7.0 buffer and were brought to a given oxidation state by titrating with concentrated stock solutions of sodium dithionite or potassium ferricyanide in 50 mM potassium phosphate buffer or addition of small amounts of the solid reagent. An excess of reagent was added to ensure maintenance of a given oxidation state during data acquisition, since HiPIPs autooxidize and/or become reduced to various extents with time (cf. Adzamli et al. 1981). Each spectrum was rescanned twice with most spectra being repeated at least twice. Data in the 250-600 nm region was obtained using a 10 mm cylindrical quartz cuvette. The absorbance of the sample was < 1.5 over this spectral region. In some cases the entire 250-600 nm region was not obtained with the same sample. In these cases, the ellipticity values between different samples in the overlapping spectral region were in reasonable agreement. In the case of suspected baseline shifts between spectra for different wavelength regions, the spectrum for the shorter wavelength region was corrected to that in the longer (e.g. near UV corrected to visible spectrum). Data in the 210-250 nm spectral region was obtained with a 1 mm cylindrical quartz cell with absorbances in this region kept to < 2. Baselines were obtained using Tris-cacodylate, pH 7 buffer. HiPIP pH difference CD spectra were obtained using sodium acetate (pH 5.1) and Tris-HCl (pH 8.7) buffers; I = 61 mM.
Visible difference absorption and CD spectra for the Rd_{ox}:FNR_{ox} complex were obtained by methods previously described for the oxidized spinach 2-Fe Fd:FNR complex (Foust et al. 1969, Shin 1973).

Redox Potentials

Oxidation-reduction potentials were obtained for most HiPIPs by two different experimental procedures. One method was the spectroelectrochemical procedure (Earl 1981) with the mediators methylviologen and ferrocyanide being used. In certain cases, (the *Ectothiorhodospira* HiPIPs) Fe EDTA was also used. The second method was the method of mixtures (see Wilson 1978 for a description). Typically, the oxidized HiPIP was titrated aerobically with concentrated solutions of ferrocyanide. The values obtained by the two methods for a given protein, under a particular set of conditions, were in reasonable agreement (± 5 mV).

Literature redox potentials used for various oxidants or conditions were: ferricinium ion +465 mV, pH 7, for pH 5 and 7.8 kinetics; (Szentrimay et al. 1977), ferricyanide +437 mV, for pH 3 kinetics, (Lappin et al. 1979), +414 mV, pH 4.8, for pH 5 kinetics, +419 mV, pH 7.8; (O'Reilly 1973), Co(phen)$_3^{3+}$ +370 mV, pH 6.5, for pH 5 and 7.8 kinetics; (Cummins and Gray 1977), Fe(III)EDTA +110 mV, pH 5; +72 mV, pH 7.8; (Kolthoff and Auerbach 1952) and *C.vinosum* HiPIP +356 mV, average value pH 7-11; (Mizrahi et al. 1980). The oxidized/semiquinone potentials used for lumiflavin were assumed to be the same as for riboflavin and were -146 mV, pH 5 and -259 mV, pH 8, for pH 7.8 kinetics (Draper and Ingraham 1968).
Data Analysis

Redox potentials determined by the method of mixtures were calculated using a version of the Nernst equation as described in Lovenberg and Sobel (1965). For the method of mixtures absorbances and HiPIP concentrations were corrected for dilution. At least four ferrocyanide additions were averaged to give the quoted values.

CD spectra were digitized using a HI-Pad digitizer (Houston Instruments) and read into a Nova II computer for calculating molar ellipticity values, averaging or subtracting spectra, and plotting.

All kinetic transients were analyzed by hand. The lines drawn for semilog plots and kobs vs. oxidant concentration plots were hand fit. The curves drawn through log kobs vs. delta E plots were least square computer fit to the Marcus exponential equation (3) with $V_{ET}$ and $\Delta G^0$ being adjustable parameters. In certain indicated cases where limited data points were available, the curves drawn were obtained using (arbitrarily) the $G^0$ values derived from the fits of analogous, more fully quantitated data sets and an estimate for $V_{ET}$ to obtain a reasonable fit.

$$k = V_{ET} \exp \left[ - \frac{\Delta G^0 + \Delta G^+(0)}{RT} \ln 2 \ln \left( 1 + \exp \left[ \frac{\Delta G^0}{\Delta G^+(0)} \right] \right) \right]$$

$G^0$ = standard free energy change for electron transfer. $\Delta G^+(0)$ is called the intrinsic barrier and is related to the internal, solvent, and atomic rearrangements that must occur prior to electron transfer.
when there is no free energy difference between reactants. R and T are the gas constant and the absolute temperature, respectively. \( V_{ET} \) is the frequency factor factor for electron transfer and is the probability of crossover from the potential surface of the reactants to products. For further discussion of the terms see Meyer et al. (1983).

The theoretical curves drawn through the ionic strength kinetic data were obtained using the equation derived by Watkins (1984) which describes the electrostatics of bimolecular reactions. The equation is discussed in Chapter 4.

Theoretical curves for the rate constant/pH profiles of flavosemiquinone oxidation are least squares fits (1/y weighted) to the equation: 
\[
kpH = (k_{HA} [H^+]) + (k_A K)/(K + [H^+]),
\]
for a single ionizable group (HA) with \( k_{HA}, k_A, \) and \( kpH \) being the second order rate constants for the fully protonated, fully dissociated, and for a given pH, respectively. \( K \) is the acid dissociation constant for the ionizable group.
FREE FLAVOSEMIQUINONE OXIDATION

Lumiflavin Semiquinone Oxidation

Disproportionation

Upon laser flash photolysis of an anaerobic solution containing Lf (see Figure 4 and Appendix A for structures) and EDTA (or methionine) a rapid transient absorbance increase is observed due to formation of Lf semiquinone, which subsequently decays by the second order disproportionation reaction (Vaish and Tollin 1970; Vaish and Tollin 1971; see also Figure 5). The rate constant for disproportionation has been found to be 5.6 times smaller for the anionic lumiflavin semiquinone (Lf⁻) than for the neutral lumiflavin semiquinone (Lf²⁺; Vaish and Tollin 1971; Edmonson et al. 1976). This was suggested to be due to electrostatic repulsion, although no direct experimental evidence for this interpretation was obtained. The effect of a change in ionic strength on the disproportionation rate constant of Lf⁻ produces only a small (<15%) increase on increasing the ionic strength (Data not shown; experiments were performed in 10 mM sodium borate and 5 mM EDTA, pH 10 at I = 51 and 551 mM. At this pH, ~98% of the Lf radical is in the anionic form). This suggests that properties other than electrical charge (e.g. electronic structure) are largely responsible for the change in disproportionation rate constant in going from the neutral to the anionic semiquinone.
Redox Proteins-pH Dependence

Addition of an oxidant, for example, an oxidized iron-sulfur protein such as \textit{C. vinosum} HiPIP, in excess of the amount of flavosemiquinone generated by the laser flash, results in a major change in flavosemiquinone decay kinetics. Semilog plots of the observed transient decays are linear over 3-4 half-lives, consistent with pseudo first order kinetics (Figure 6). No HiPIP reduction or alteration in the flavosemiquinone disproportionation reaction is observed in the absence of flavin or in the presence of reduced HiPIP, respectively. The $k_{\text{obs}}$ values increase linearly with increasing oxidized HiPIP concentrations at all pH values (Figure 7). It should be noted that due to the low reactivity of HiPIP with LfH$^*$, the disproportionation reaction, at low protein concentrations, competes effectively with HiPIP oxidation of the flavin semiquinone. This requires Lf semiquinone oxidation at low pH values to be measured at high HiPIP concentrations resulting in poorer quality of the data. A large increase is found in flavosemiquinone reactivity between pH 5 and 11 (~ 60 fold) as is indicated by the data in Figure 8. The pK (8.4) determined from the change in apparent second order rate constant (electrostatically uncorrected) as a function of pH is in good agreement with the LfH$^*$ N-5 deprotonation pK previously measured (8.36, Lf, Vaish and Tollin 1971; 8.4, Lf-3-acetate; Ehrenberg et al. 1967).

The reduction of \textit{C. vinosum} HiPIP by ascorbate, dithionite, and ferrocyanide at pH 7.3 are independent of ionic strength ($I = 10$-200 mM; Mizrahi et al. 1976). At pH 10, no (<15%) ionic strength effect on the rate constant for the Lf semiquinone reaction is observed. Thus,
neglecting to electrostatically correct the apparent second order rate constant to infinite ionic strength would be expected to have a negligible effect on the apparent pK for the reaction. To investigate the possibility that the large reactivity difference between neutral and anionic flavosemiquinones is unique to HiPIP, the pH dependence of Lf semiquinone oxidation by two other types of iron-containing redox proteins, *C. pasteurianum* rubredoxin and tuna heart cytochrome c were examined. As shown in Figure 8, the data for these proteins, normalized to those of *C. vinosum*, follow an ionization curve with the same pK as that seen for HiPIP. This then further supports the assignment of this pK to the N-5 position of LfH* and suggests that any pH-dependent changes in protein reactivity are quite small. In pH studies with other reductants, *C. vinosum* HiPIP has been found to exhibit a small pH dependence (at pH values < 7, electrostatically uncorrected; Mizrahi et al. 1976); while *C. pasteurianum* rubredoxin reactivity was found to be pH independent (pH 6.3-8, electrostatically uncorrected; Jacks et al. 1976). Monomeric cytochrome c is known to undergo an alkaline conformational change (pK 9; Gupta and Koenig 1971) in which only one conformer is reactive with ascorbate (Greenwood and Palmer 1965). This phenomenon may explain the slightly poorer fit of the cytochrome data points. However since the pH studies were carried out using the dilution method, the time-dependent alkaline conformational change may be minimized (Greenwood and Palmer 1965).

Inasmuch as LfH* must lose a proton upon oxidation (Figure 4), the observed pH dependence could conceivably represent a rate-limiting
Figure 6. Anaerobic lumiflavin semiquinone oxidation by oxidized *C. vinosum* HiPIP at pH 8.0.

Solution conditions were sodium phosphate buffer, 10 mM EDTA, pH 8.0 and I = 0.24 M with a 35 micromolar oxidized lumiflavin and 24 micromolar HiPIP.
Figure 7. The $k_{obs}$ versus C. vinosum HiPIP concentration plots for lumiflavin semiquinone oxidation as a function of pH.

pH 5.0 (o), pH 6.0 (square), pH 7.0 (diamond), pH 7.5 (tri), pH 8.0 (star), pH 8.25 (triangle), pH 8.5 (bullet), pH 9.0 (circle), pH 10.0 (cross), pH 11.0 (triangle-up)
Figure 8. Dependence on pH of second order rate constant for Lf semiquinone oxidation by iron-containing redox proteins.

The solid line is a theoretical curve obtained from a non-linear least squares (1/y weighted) to fit to the *C. vinosum* HiPIP data (*●*). The parameters for the fit are $k_{HA} = 1.2 \times 10^7$ M$^{-1}$s$^{-1}$, $k_A = 6.5 \times 10^6$ M$^{-1}$s$^{-1}$, and $pK = 8.4$. The data for *C. pasteurianum* rubredoxin (*○*) and tuna heart cytochrome *c* (*▲*) were normalized to the HiPIP fit. The parameters for the data fits with these latter proteins were $k_{HA} = 1.3 \times 10^7$ M$^{-1}$s$^{-1}$, $k_A = 4.5 \times 10^6$ M$^{-1}$s$^{-1}$ and $pK = 8.3$; and $k_{HA} = 1.5 \times 10^7$ M$^{-1}$s$^{-1}$, $k_A = 1.5 \times 10^6$ M$^{-1}$s$^{-1}$; and $pK = 8.1$, respectively.
Figure 8. Dependence on pH of second order rate constant for Lf semiquinone oxidation by iron-containing redox proteins.
deprotonation reaction. However, since the reactions were carried out in buffered solutions, and no major effect of carrying out the reaction in D$_2$O was observed, (both reactions were carried out a pH 5 with no correction made for the effect of D$_2$O on pH) this possibility is unlikely. Instead, the pH dependence more probably reflects the increase in concentration of the intrinsically more reactive Lf$^\cdot$ as the pH is increased. This explanation has been previously given for the strongly pH-dependent dioxygen/flavosemiquinone reaction (Vaish and Tollin 1971; Edmondson et al. 1977).

Inorganic Oxidants—pH Dependence

The large Lf semiquinone reactivity ratio displayed by the three different redox proteins is striking, considering that, except for dioxygen, non-biological oxidants have previously been found to show only small reactivity differences between neutral and anionic Lf semiquinones. More specifically, ferricyanide oxidation increases only slightly (~50%) with pH (Table 2; see also Vaish and Tollin 1971; Edmondson et al. 1976); and p-benzoquinone is only ~5 fold faster with Lf$^\cdot$ (Vaish and Tollin 1971). In order to investigate this further, several additional small molecule oxidants have been examined (Table 2). Like ferricyanide, the ferricinium ion exhibits a negligible pH effect. However, Fe(III)EDTA and Co(phen)$_3^{3+}$ both show a pronounced pH effect on Lf semiquinone oxidation. Since Fe(III)EDTA (actually FeEDTA(H$_2$O)~$^-$ has an acid dissociation (pK 7.58) yielding the species FeEDTA(OH)$^2$~ which can undergo dimerization to form a $\eta$-oxo species, [(FeEDTA)$_2$O]$^4$~ (Gustafson and Martell 1963), the pH results with this
reactant must be viewed with caution. A recent interpretation of the pH dependence of the oxidation of reduced spinach [2Fe-2S] ferredoxin by Fe(III)EDTA, however, has suggested that the small decrease in reactivity with increasing pH (pK 7.2) originates with Fe(III)EDTA (Armstrong and Sykes 1978). The observed change in reactivity is in the opposite direction and is consistent with the pH effects seen with Co(phen)$_3^{3+}$ (which has no protonation site) and the redox proteins. This suggests that the pH effects observed with Fe(III)EDTA and Co(phen)$_3^{3+}$ have the same origin as stated above, i.e. an increase in concentration of the more reactive Lf$^-$ species. The lack of substantial pH effects with ferricyanide (Vaish and Tollin 1971) and ferricinium ion could be interpreted as being due to these oxidants reacting at a site removed from N-5. Another possible explanation is that the rate constants for the reaction of these oxidants, with LfH$, are approaching a diffusion-controlled limit, so that no substantial increase can be observed upon increasing the concentration of the intrinsically more reactive Lf$^-$ form.

Effect of Inorganic Oxidant Redox Potential

Analysis of the pH 7.8 rate constant data (where a substantial amount of Lf$^-$ exists) as a function of $\Delta E$ (Figure 9), demonstrates that these data can be fit to the free energy relationship of Marcus (1968) which has previously been used to systematize electron transfer reactions between free or protein-bound flavosemiquinones and various types of redox proteins (Meyer et al. 1984; Tollin et al. 1984). Obviously, more oxidants need to be examined to fully quantitate the
Table 2. Apparent second order rate constants (M\(^{-1}\)s\(^{-1}\)) for Lf semiquinone oxidation by various one-electron oxidants at pH 5 and 7.8.

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>pH 5</th>
<th>pH 7.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>ferricinium ion</td>
<td>1.6x10(^9)</td>
<td>1.5x10(^9)</td>
</tr>
<tr>
<td>ferricyanide</td>
<td>5.7x10(^8)</td>
<td>8.7x10(^8)</td>
</tr>
<tr>
<td>Co(phen)(_3)(^{3+})</td>
<td>9.3x10(^6)</td>
<td>6.7x10(^8)</td>
</tr>
<tr>
<td>Fe(III)EDTA</td>
<td>7.7x10(^5)</td>
<td>3.7x10(^7)</td>
</tr>
</tbody>
</table>
Figure 9. Semilog plot of the second order rate constant ($k$) vs. the difference in redox potential ($\Delta E$) for oxidation of lumiflavin semiquinone.

Oxidants are ferricinium ion (A), ferricyanide (B), Co(phen)$_3^{3+}$ (C), and Fe(III)EDTA (D), at pH 5 (○), and 7.8 (●). The curve drawn through the pH 7.8 data represents a non-linear least squares fit to the Marcus exponential equation with the parameters for the fit as indicated. For the Co(phen)$_3^{3+}$/Fe(III)EDTA pH 5 data the curve drawn was obtained by assuming the same $\Delta G^\ddagger(0)$ value and adjusting the $V_{ET}$ value to get a reasonable fit.
Figure 9. Semilog plot of the second order rate constant (k) vs. the difference in redox potential (ΔE) for oxidation of lumiflavin semiquinone.
relationships shown in Figure 9. It is interesting, however, that the relative order of reactivity, ferricyanide > Co(phen)$_3^{3+}$ > Fe(III)EDTA, is the same as seen for the rate constants (electrostatically corrected to infinite ionic strength) for flavodoxin semiquinone oxidation (Tollin et al. 1984) and for horse heart cytochrome c oxidation (Wherland and Gray 1976) by these reactants.

The fact that the Fe(III)EDTA/Co(phen)$_3^{3+}$ pH 5 data (where LfH$^+$ predominates) can be fit to a parallel, downward-shifted curve relative to the pH 7.8 curve, is consistent with a decreased flavosemiquinone reactivity (reflected in the smaller $\bar{E}T$ value which is the limiting rate constant as $E$ approaches infinity), with no drastic change in the solvent or nuclear rearrangements (reflected in $G^+ (0)$) required to reach the transition state for electron transfer. At pH 5, a clear distinction in the intrinsic reactivity (i.e. beyond redox potential effects) between ferricyanide (as well as the ferricinium ion) and Co(phen)$_3^{3+}$/Fe(III)EDTA is apparent. A rationalization for precisely this same ordering of the relative reactivities of these reactants towards flavodoxin and cytochrome c, based on their hydrophobic, steric, and degree of pi-donor ligand properties, has previously been given (Wherland and Gray 1976; Tollin et al. 1984). The investigation of other oxidants is required to support the above speculations.

Effect of HiPIP Redox Potential

At pH 7 (where LfH$^+$ predominates), a redox potential dependence on the second order rate constant for Lf semiquinone oxidation is observed when HiPIPs with different redox potentials are used as
oxidants. The data give reasonable least squares error fits (Figure 10) to the exponential equation of Marcus (1968). The values of $V_{ET}$ and $\Delta G(0)$ are both smaller for the HiPIP fit than the pH 5.0 data with inorganic oxidants (see above). Due to the limited number of inorganic oxidants investigated, interpretation of the differences in these parameters for the two fits is at present unwarranted. However, fits of the second order rate constants for the semiquinone oxidation by a large number of homologous $g$-type cytochromes of different redox potentials yield similar parameter values as observed with the HiPIP reaction. The small differences observed were suggested to be a consequence of differential solvent exposure of the redox center in the two types of proteins (Meyer et al. 1983).

Oxidation of 5-deazariboflavin semiquinone by HiPIP has also been examined. The fit shown (Figure 10) to the data was calculated by arbitrarily using the $\Delta G^\pm(0)$ values derived from the Lf semiquinone fit and an estimate for $V_{ET}$. The apparent second order rate constants for this reaction are probably approaching a diffusion-controlled limit. The apparently greater intrinsic reactivity of 5-deazariboflavin semiquinone cannot be rationalized at present in light of the lack of information available on the electron spin density distribution of this flavin (see below).

In addition to Lf semiquinone reduction of HiPIP, a slower HiPIP reduction reaction is observed. It has been assumed that this slower reaction represents reduction by fully reduced flavin arising from the disproportionation of laser-flash-generated flavosemiquinone (cf. Figure 5, page 40). The apparent second order rate constant for
this reaction, at pH 7.0, also demonstrates a dependence on HiPIP redox potential (Figure 10). The lower $\nu_{ET}$ for this reaction, as compared to the Lf semiquinone reaction, may arise from differences in charge and electronic structure for the two reduced flavin species (cf. Figure 4, page 34).

**Effect of Unpaired Electron Distribution**

Recently, it has been shown to be possible to quantitate not only redox potential effects, but also steric and electrostatic influences on free and protein-bound flavosemiquinone oxidation by homologous cytochromes $c$ (Meyer et al. 1983; Meyer et al. 1984; see also flavodoxin semiquinone oxidation by HiPIP). The observed differences in anion vs. neutral semiquinone reactivity indicate that, at least for free flavins, factors in addition to redox potential, presumably electronic, also play an important role in influencing electron transfer. These electronic effects can be quantitated by determining the limiting rate constants for the two flavosemiquinone species (see below).

As mentioned above (page 37), an alteration in unpaired electron spin density distribution occurs in going from the neutral to the anionic flavosemiquinone. If this had a major influence on the reactivity of these species then the involvement of positions C-4a, C-6, and C-8 in semiquinone electron transfer would be indicated. In anionic flavosemiquinones, the largest increase in spin density occurs at C-4a (5-fold) with the N-5-C4a region containing ~50% of the total spin density cf. Edmondson and Tollin 1983 and references cited
Figure 10. Semilogarithmic plot of apparent second order rate constants for flavosemiquinone and flavohydroquinone oxidation by HiPIP as a function of the difference in midpoint redox potentials (pH 7.0).

The letter s beside each point identifies the protein source as given in Appendix B. The solid lines represent least squares fit to the Marcus exponential equation using the parameters indicated (▲) HiPIPs + 5-d-Rf semiquinone; (●) HiPIPs + lumiflavin semiquinone, (x) HiPIPs + fully reduced lumiflavin.
Figure 10. Semilogarithmic plot of apparent second order rate constants for flavosemiquinone and flavohydroquinone oxidation by HiPIP as a function of the difference in midpoint redox potentials (pH 7.0).
therein). In the reaction of flavohydroquinone with oxygen, a C-4a adduct is formed (cf. Favaudon 1977 and references therein). If the oxygen/flavosemiquinone reaction also occurs at position C-4a, the increased spin-density in the anion at this position would be consistent with its greater reactivity. Another possible reason for the increased rate constant for oxidation of the anion semiquinone by dioxygen is its lower redox potential (-146 vs. -392 mV at pH 5 and 11, respectively, for riboflavin; Draper and Ingraham 1968).
Flavosemiquinone Analog Oxidation

C. vinosum HiPIP as the Oxidant

The pH dependencies of oxidation of other flavosemiquinone analogs by C. vinosum HiPIP display similar increases in reactivity with increasing pH with the parameters for the fits summarized in Table 3. The pH range (5-10) studied was determined by HiPIP stability and decreased flavosemiquinone yield at high pH (presumably due to EDTA ionization and/or flavoquinone N-3 ionization see page 36). For most analogs, the lower end of the ionization curve is not well defined, thus decreasing the accuracy in the limiting second order rate constant for the neutral flavosemiquinone. This fact also limits the accuracy in the determined pK values. The estimated error in these latter numbers is ±0.3.

Semiquinone pK Values

Except for the 8-alpha-substituted analogs (8-alpha-substituent pK ~5; Merrill and McCormick 1978), no additional ionizable groups occur in the various substitutions, therefore, by analogy to Lf, the observed pH dependence is attributed to the ionization of the N-5 position of the neutral flavosemiquinones. This conclusion is further supported by the fact that 5-deazariboflavin (N-5 is replaced by CH) does not exhibit any major change (< 50 %) in reactivity with HiPIP between pH 5 and 10. The pK value for riboflavin (Rf) semiquinone is
Table 3. Computed second order rate constants for anionic and neutral flavosemiquinone oxidation by *C. vinosum* HiPIP and N-5 pK values for various flavin analogs.

<table>
<thead>
<tr>
<th>Flavin Analog</th>
<th>$k_{HA} \times 10^{-7} \cdot M^{-1} \cdot s^{-1}$</th>
<th>$k_{A} \times 10^{-7} \cdot M^{-1} \cdot s^{-1}$</th>
<th>$K_M(\text{pK})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-MI</td>
<td>1.2</td>
<td>66</td>
<td>$1.9 \times 10^{-8}(7.7)$</td>
</tr>
<tr>
<td>Lf</td>
<td>1.8</td>
<td>65</td>
<td>$4.4 \times 10^{-9}(8.4)$</td>
</tr>
<tr>
<td>Rf</td>
<td>1.2</td>
<td>44</td>
<td>$4.3 \times 10^{-9}(8.4)$</td>
</tr>
<tr>
<td>7-ClRF</td>
<td>0.9</td>
<td>52</td>
<td>$4.1 \times 10^{-8}(7.4)$</td>
</tr>
<tr>
<td>8-ClRF</td>
<td>0.7</td>
<td>42</td>
<td>$5.9 \times 10^{-8}(7.2)$</td>
</tr>
<tr>
<td>7,8-diClRF</td>
<td>0.7</td>
<td>40</td>
<td>$3.8 \times 10^{-7}(6.4)$</td>
</tr>
<tr>
<td>8 MP-T</td>
<td>1.6</td>
<td>25</td>
<td>$2.4 \times 10^{-7}(6.6)$</td>
</tr>
<tr>
<td>8 MP-S</td>
<td>0.2</td>
<td>15</td>
<td>$9.3 \times 10^{-8}(7.0)$</td>
</tr>
</tbody>
</table>

1 Abbreviations as given in Appendix A.
found to be the same as that of LfH', in agreement with a previous report (Vaish and Tollin 1971). In some cases, the pK value varies according to the expected inductive effects of the various exocyclic substituents (\(-\text{CH}_3 > -\text{H} > -\text{Cl}\); Table 3). The lower pK value for 7,8-diClRF vs. the monochloro derivatives is also consistent with the expected greater inductive effect of two chlorine substitutions. Moreover, in agreement with previous work (Edmondson et al. 1977), 8-alpha-substitution decreases the N-5 pK. Oxidation of the thioether sulfur of 8 MP-T to the sulphone increases the N-5 pK. This sulfur oxidation effect is opposite to the decrease in N-1 pK values observed for the oxidized state of other 8-alpha-sulfur-substituted analogs (Falk et al. 1976). The reason for the difference is unclear.

Effect of Flavin Redox Potential on Flavosemiquinone Oxidation

A comparison of the limiting anionic flavosemiquinone rate constants (which are the most accurately determined) indicates that there are differences (albeit small) between the various analogs (Table 3). Based upon absolute rate constant values, there appears to be little steric hindrance introduced upon the substitution of the C-7 and C-8 hydrogen atoms for methyl groups (10-MI vs. Lf; Table 3). However, examination of rate constant vs. redox potential difference plots (Figure 11) indicates that 10-MI is in fact intrinsically more reactive than Lf, presumably due to steric effects.

The redox potentials used in the above analysis are the two-electron potentials (Appendix A) in all cases, except for 5-dRF, for which the oxidized/semiquinone redox potential value was used. The
difference between the oxidized/fully-reduced and the oxidized/semiquinone potentials for Rf is 42 mV at pH 11 (Draper and Ingraham 1968). This is relatively small, and it is assumed to be approximately the same for all of the analogs used.

It is of interest that the intrinsic barrier ($\Delta G^\ddagger(0) = 3.8 \text{ Kcal mol}^{-1}$) for the fit shown is nearly identical to that obtained (3.7 Kcal mol$^{-1}$) for the pH 7 Lf semiquinone reaction with a large number of HiPIPs of different redox potentials (Figure 10). The similarity in $G^\ddagger(0)$ values suggests no major difference in the structural rearrangements required to reach the transition state for semiquinone oxidation with variations in the flavin or HiPIP structures. The much larger $\nu_{PT}$ value for the anionic semiquinones ($1.4 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) (compared to $9.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7 for the reaction of Lf semiquinone with various HiPIPs) is consistent with the greater intrinsic reactivity of this form of the semiquinone.

Substitution of the N-10 methyl group with a ribityl side chain results in a $-32\%$ decrease in rate constant (Lf vs. Rf). An essentially identical average decrease in rate constant ($-30\%$) between these two flavin analogs has been observed for a group of homologous $c$-type cytochromes (Meyer et al. 1984), and has been attributed to steric interference by the ribityl side chain. The similar magnitude for the decreased Rf semiquinone reactivity observed with the two types of redox proteins, suggests that we are observing primarily a flavin-dependent property. An additional contribution to the reactivity decrease could be that the highly solvent hydrogen-bonded (polyhydroxyl) ribityl side chain results in a longer rotational
Figure 11. Semilog plot of the second order rate constant (k) vs. difference in redox potential (ΔE) for oxidation of various flavosemiquinone analogs by *C. vinosum* HiPIP (▲) or ferricyanide (●).

The analogs are 7,8-diClRf (A), 7-ClRf (B), 8-ClRf (C), 10-MI (D), Lf (E), Rf (F), and 5-dRf (G) (Abbreviations as given in Table 1). The ferricyanide rate constants are for reactions performed at pH 3 (where the neutral flavosemiquinone predominates), while the HiPIP rate constants are the fitted anionic semiquinone values (Table 3).
$\nu_{ET} = 1.6 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}$

$\Delta G^0 (0) = 7.0 \text{ Kcal mol}^{-1}$

$\nu_{ET} = 1.4 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$

$\Delta G^0 (0) = 3.8 \text{ Kcal mol}^{-1}$

Figure 11. Semilog plot of the second order rate constant (k) vs. difference in redox potential (ΔE) for oxidation of various flavosemiquinone analogs by *C. vinosum* HiPIP (▲) or ferricyanide (■).
correlation time for the flavosemiquinone leading to a decreased probability of forming an optimal orientation for electron transfer. The complicated ESR hyperfine splitting observed with Rf semiquinone has been attributed to such a strong ribityl side chain interaction with the solvent (cf. Edmondson and Tollin 1983). On this basis, analogs with substituents which could also strongly interact with the solvent (e.g. electrically charged groups) would be expected to have a lower intrinsic reactivity. Indeed, this is what is observed for 8-alpha-substituted analogs (see below) as well as for FMN (Chapter 4; Meyer et al. 1984). In the latter case, part of the observed decrease is due to the slightly higher N-5 pK value for FMN (Meyer et al. 1984). This interpretation is amenable to further experimental testing by using flavin analogs with N-10 substituents which possess decreased hydrogen-bonding capabilities but which have similar steric properties to Rf, and by using altered solvents (e.g. D₂O).

The small size difference between chlorine atoms and methyl groups would not be expected to induce much of a steric effect. The observed differences in rate constant among the chlorine-substituted analogs, then, are probably due to inductive and, to a lesser extent, resonance effects of chlorine on electron density distribution. If this interpretation is correct, then the larger than expected rate constant for 7-ClRf vs. Rf may be due to a redistribution of electron density from predominantly the C-8 and N-10 positions towards the C-7 position. Such a change in electronic structure would be expected to increase the rate of flavosemiquinone oxidation if this reaction was occurring near the C-6-C-7 region of the dimethyl benzene ring. The
fact that 8-ClRf does not show this increase (relative to Rf) suggests a positional effect for chlorine substitution. Although the electronic states of oxidized and semiquinone flavin are by no means identical, it is worth noting that an opposing positional effect of C-7, C-8 methyl substitution on the fluorescence properties of flavoquinone has been reported (Visser and Muller 1979). The possibility of secondary effects resulting from the electron density redistribution, i.e. a change in the average ribityl side-chain conformation, also exists. It is interesting that a similar increase in rate constant for 7-ClFMN-substituted flavodoxin semiquinone oxidation by horse heart cytochrome c over that for native flavodoxin has been observed (Simonsen and Tollin 1983).

The slower rate constant for 8-α-PMP, a model for some naturally-occurring covalently-linked flavins in flavoproteins, may be due to steric interference and solvation effects from the bulky, electrically charged 8-alpha-substituent and in part to repulsive electrostatic effects. Oxidation of the sulfur results in a further decrease in rate constant. This effect suggests a possible direct involvement of the sulfur atom in flavosemiquinone oxidation. This deserves further investigation, inasmuch as it may be relevant to flavoenzyme mechanisms.

Potassium Ferricyanide as the Oxidant

Due to the larger uncertainties in the neutral flavosemiquinone/HiPIP rate constants obtained from the data fits (see above), we have investigated the oxidation of neutral flavosemiquinones
by ferricyanide as an approach to examine their relative reactivities. The rate constants for oxidation of the various analogs demonstrate a dependence on the redox potential difference for the reaction (Figure 11). Linear free energy relationships between the $V_{\text{max}}$ for the NADPH steady-state reduction of ferricyanide catalysed by flavoproteins, substituted with different midpoint potential flavins, have been previously shown to exist (Light and Walsh 1980; Zanetti et al. 1983). The different analog effects described for the anionic flavosemiquinone/HiPIP data are not as evident with the structurally simpler oxidant ferricyanide. This may be due to a different reaction site on the flavin, the lack of steric hindrance with ferricyanide, and/or differences between the anionic and the neutral flavosemiquinones. A similar situation has been observed previously with FMN and flavodoxin (Tollin et al. 1984) in which reactivity differences among various $c$-type cytochromes are much smaller with the former than with the latter. This was attributed to the simpler structure of FMN which results in fewer constraints for the attainment of a productive alignment between the reactants (see also Flavodoxin Semiquinone Oxidation by HiPIPs). Such an explanation may well apply to the ferricyanide-HiPIP comparison.
Conclusions

Free anionic flavosemiquinones have a higher intrinsic reactivity with a variety of one-electron oxidants than do neutral flavosemiquinones. The origin of this increased reactivity is probably the different electron spin-density distributions between the two semiquinone forms. Although the biochemical reasons for the existence of covalently-linked flavins in certain flavoproteins is presently unknown, these enzymes are found to produce anionic flavosemiquinones at physiological pH (Edmondson et al 1981). Thus, they may be utilizing the increased reactivity of this form of one-electron reduced flavin as a component of their reaction mechanism. The rate constants for the oxidation of the different protonic forms of various free flavosemiquinone structural analogs by redox proteins and non-biological oxidants can be correlated redox potential difference for the reaction using the Marcus free energy relationship. On this basis of the results, it can be concluded that the C-4a and the N-5-dimethylbenzene ring regions play an important role in the mechanism of flavosemiquinone oxidation.
FLAVODOXIN OXIDATION BY HIPIP

The electron transfer reaction between flavodoxin semiquinone and all HiPIPs was biphasic over the time range available. The fast phase gave linear semilogarithmic plots (cf. Figure 12) and comprised typically 70-80% of the total reaction observed. This was true for all HiPIP concentrations studied, for all ionic strengths, and for each HiPIP source (cf. Figure 13). The slow reaction rate constant was found to be concentration independent with a first order rate constant (~0.05 s\(^{-1}\)) that did not vary substantially from source to source among various HiPIPs (Cheddar 1984, personal communication), as well as \(\alpha\)-type cytochromes (Watkins 1984). In another protein/protein reaction involving HiPIP and \(\alpha\)-type cytochromes biphasic kinetics have been observed; however the heterogeneity was found to be cytochrome dependent (Mizrahi and Cusanovich 1980). This suggests, in contrast to an earlier interpretation (Simonsen et al. 1982), that the slow reaction may reside with flavodoxin. Obviously, further experimentation is required to determine if this is indeed the case. For the present study, however, it will be assumed that the slow reaction is associated with a flavodoxin-dependent process and only the fast reaction, which represents flavodoxin semiquinone oxidation (Simonsen et al. 1982), will be discussed further.
Figure 12. Typical fast phase reaction trace for oxidation of flavodoxin semiquinone by *C. vinousum* HiPIP

*C. vinousum* HiPIP concentration 26 micromolar, pH 7.2, and $t^{1/2} = 0.454$, 23.5°C, 580 nm.
Figure 13. The dependence of $k_{obs}$ for flavodoxin semiquinone oxidation on C. vinosum HiPIP concentration at different ionic strengths. 

$I^{1/2} = 0.7$ (x), $I^{1/2} = 0.454$ (o), $I^{1/2} = 0.312$ (e), $I^{1/2} = 0.24$ (A), $I^{1/2} = 0.17$ (B), $I^{1/2} = 0.12$ (v); units M$^{1/2}$
Ionic Strength Dependence

Plots of the logarithm of the apparent second order rate constant versus the square root of the ionic strength are shown in Figure 14. The theoretical curves shown in Figure 14 were obtained from the electrostatic equation for bimolecular reactions of Watkins (Watkins 1984). This equation has been successfully used to fit the kinetic-ionic strength data of an analogous reaction system involving flavodoxin semiquinone and various c-type cytochromes (Tollin et al. 1984). The equation (4) treats the reactants, flavodoxin semiquinone and oxidized HiPIP, as parallel disks (of a radius $\rho$), with discrete point charges $(z_1, z_2)$ equal to the total charge in this region on the reactants which results in an electrostatic potential energy, $V_{ii}$.

$$\ln k(I) = \ln k_\infty - V_{ii} X(I)$$  \hspace{1cm} (4)

In equation (4), $k(I)$ is the observed rate constant at ionic strength $I$, $k_\infty$ is the rate constant at infinite ionic strength, $V_{ii}$ is as defined above, and $X(I)$ is an ionic strength dependent term equal to $(1 + k\epsilon)^{-1} \exp(-k\epsilon)$ with $k\epsilon$ as defined above and $\epsilon = 0.3295 I^{1/2}$. From the data analysis using equation (4), values for two parameters are obtained for a given value, $V_{ii}$ (which can yield the charge product $(z_1z_2)$ for the reactants, see equation 5 below) and the rate constant at infinite ionic strength ($k_\infty$, see Table 4).
Figure 14. Ionic strength dependence of the apparent second order rate constants for electron transfer from flavodoxin semiquinone to oxidized HiPIP.

Curves represent theoretical fits of the data to equation (4) see text for details. Curves for the different HiPIPs labeled as in Table 4.
From the $V_{ii}$ values obtained for the fits shown in Figure 14 and equation (5) the charge product ($Z_1 Z_2$) can be calculated.

$$V_{ii} = \alpha \epsilon^{-2} D_e^{-1} Z_1 Z_2 r_{12}$$  \hspace{3cm} (5)

The parameters are: $\alpha$, a constant = 128.47; $\epsilon$, (although this value could have been varied, it was taken as 8.25 Å to reduce the number of fitted parameters and on the average this value gave the best least squares error fit. Moreover, this value is similar to that used (7.25 Å) in the flavodoxin/cytochrome $c$ study; Tollin et al. 1982.); $D_e$, the effective dielectric constant within the interaction domain (a value of 15 was used; this value was calculated from the observed $V_{ii}$ for $C.\ vinosum$ HiPIP and assuming a charge at the interaction site on HiPIP of $-1$ which is consistent with the expected charge at the HiPIP reaction site with the iron hexacyanides Mizrahi et al. 1980; see also page 22. A value of 15 for $D_e$ is also consistent with the hydrophobic nature of this suggested site as well as the possible displacement of water at the interaction domain, see page 26.); and $r_{12}$, the distance between the electron transfer groups within the reactive complexes (a value of 8.0 Å was used, the distance of closest approach of atoms of the [4Fe-4S] cluster to the surface is ~ 4.5 Å, plus the Van der Waals radius, 3.5 Å; Carter et al. 1974a, see page 19).

For the calculation of $Z_1$, the charge at the interaction domain of each HiPIP, a value of $-4$ for the flavodoxin interaction domain ($Z_2$) was used. This value was obtained from the putative flavodoxin:cytochrome $c$ complex and is in qualitative agreement with the negative charge observed for flavodoxin semiquinone in reactions with a number of charged oxidants (Simonsen et al. 1982, Tollin et al.
The value for the charge at the interaction site on the various HiPIPs ($Z_1$) is given in Table 4. The sign of the charge, in all cases, is the same as the protein net charge, although the magnitudes are quite different. This is in contrast to a cytochrome/flavodoxin study (Tollin et al. 1984) in which charge signs different from the protein net charge were observed in some cases. The sign of the HiPIP charge is also consistent with the local interaction site proposed for the iron hexacyanides (Mizrahi et al. 1980; page 22) and cytochrome c (Aprahamian and Feinberg 1981, Mizrahi and Cusanovich 1980). At this rather hydrophobic site, the delocalized negative charge of the cluster has been suggested to dominate electrostatic interactions with charged amino acid side chains in sequence positions 46-49, 63-65, and 78-82 and at greater distances 31-34 and 16-17 having a smaller role (Mizrahi et al. 1980). The magnitude for the $Z_1$ values are also in good agreement with the iron hexacyanide site. For example, $T.$ roseopersicina HiPIP, assuming all other parameters are constant, demonstrates ~40% of the charge at the interaction domain as does $C.$ vinosum HiPIP, even though the $T.$ roseopersicina HiPIP net charge could be no smaller than 75% that of $C.$ vinosum (this lower limit assumes that the two additional histidines in $T.$ roseopersicina HiPIP are fully protonated at pH 7.2; which seems unlikely). The lower apparent charge at the interaction domain for $T.$ roseopersicina can be rationalized in terms of the iron hexacyanide site by the presence of HIS 49 which lies within 5 Å of the $S^*_4$ atom of the cluster (based on the position of MET 49 in the $C.$ vinosum HiPIP structure) and which would be expected to
Table 4. Effect of ionic strength on the oxidation of flavodoxin semiquinone HiPIP.

|                | $E_m, 
u (v)$ | Net Charge $^a$ | Number of Histidine Residues | Vii (Kcal/mole) | $\phi$ (A) | $z_1^b$ | $k_\infty \times 10^4$ (M$^{-1}$s$^{-1}$) |
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C. vinosum (A)</td>
<td>0.36</td>
<td>-4</td>
<td>1</td>
<td>+4.05</td>
<td>8.25</td>
<td>-1.0</td>
<td>18.7</td>
</tr>
<tr>
<td>C. gracile (B)</td>
<td>0.35</td>
<td>-6</td>
<td>2</td>
<td>+4.77</td>
<td>8.25</td>
<td>-1.2</td>
<td>10.0</td>
</tr>
<tr>
<td>T. pfennigii (C)</td>
<td>0.35</td>
<td>-8</td>
<td>4</td>
<td>+7.20</td>
<td>8.25</td>
<td>-1.8</td>
<td>9.1</td>
</tr>
<tr>
<td>T. roseopersicina (D)</td>
<td>0.34</td>
<td>-5</td>
<td>3</td>
<td>+1.69</td>
<td>8.25</td>
<td>-0.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Rp. gelatinosa (E)</td>
<td>0.33</td>
<td>+4</td>
<td>1</td>
<td>-6.01</td>
<td>8.25</td>
<td>+1.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Rs. tenue 3761 (F)</td>
<td>0.30</td>
<td>+3</td>
<td>0</td>
<td>-5.30</td>
<td>8.25</td>
<td>+1.3</td>
<td>0.83</td>
</tr>
<tr>
<td>Rs. tenue 2761 (G)</td>
<td>0.30</td>
<td>+2</td>
<td>0</td>
<td>-5.72</td>
<td>8.25</td>
<td>+1.4</td>
<td>0.79</td>
</tr>
<tr>
<td>E. vacuolata iso-1 (H)</td>
<td>0.26</td>
<td>-4</td>
<td>3</td>
<td>+5.30</td>
<td>8.25</td>
<td>-1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>E. vacuolata iso-2 (I)</td>
<td>0.15</td>
<td>-7</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.06$^c$</td>
</tr>
</tbody>
</table>

$^a$ Data used to calculate from Table 1 and Meyer 1984, personal communication. The minus one cluster charge is included. The value does not include the N and C$^-$ termini charges. It was assumed that all histidines were unprotonated, but this is probably not the case.

$^b$ The values for the parameters in equation (5) used to calculate $z_1$ are given in the text.

$^c$ Since only two ionic strength values for this HiPIP could be obtained, the $k_\infty$ infinity was estimated.
contribute to the neutralization of the cluster's negative charge (see pH Perturbation of HiPIP Visible CD). Further, the similarity in the magnitude of $Z_1$ for \textit{C. vinosum} and \textit{C. gracile} HiPIPs is consistent with no expected charge differences between the proteins in the proposed interaction domain (based on sequence homologies). \textit{T. pfennigii} HiPIP has two additional charged groups (as compared with \textit{C. vinosum}) near the interaction site, HIS 82 and GLU 35 at a greater distance. These residues can account for the greater negative charge observed for this protein. \textit{R. gelatinosa} HiPIP, which demonstrates a $Z_1$ value equal to +1.5, has two additional lysine residues (LYS 31 and LYS 82) near the proposed iron hexacyanide interaction site compared to \textit{C. vinosum} HiPIP. At a greater distance LYS 52 (which is ASP 52 in \textit{C. vinosum}) can also contribute to the observed charge of the interaction domain.

The two \textit{R. tenue} strains have LYS 64 near the proposed interaction site. Since these proteins represent the smallest and most divergent HiPIPs to be sequenced (Meyer 1984, personal communication), the location of deletions in the sequence are not known with much certainty. It is therefore possible that ARG 14, LYS 15 and/or LYS 38 (present alignment, Table 1) could occupy positions in the structure which could contribute to the charge at the interaction site (Table 4). In addition, loss of the negative charge at position 52 relative to \textit{C. vinosum} (GLY 52 for ASP 52) relative to \textit{C. vinosum} could also contribute to the observed charge difference. Although \textit{E. vacuolata} iso-l-HiPIP does not have any additional charges at the proposed interaction site, it does have ARG 33 substituted (Meyer 1984, personal communication) which could account for its slightly greater negative
value for $Z_1$ (Table 4). These arguments receive some support from similarities observed with FMN semiquinone oxidation by HiPIP (see below). Computer modeling studies of the electrostatic fields of the various HiPIPs (based on hypothetical three dimensional structures using the *C. vinosum* HiPIP structure as a starting point) and the results of high resolution $^1$H-NMR experiments presently in progress (Cusanovich 1984, personal communication) may provide further support.
Effect of Redox Potential

The $k_\circ$ values (i.e. the rate constant at which all charge effects are presumably screened) obtained from the fits in Figure 14 were used to examine their dependence on the redox potential difference for the reaction (Figure 15). A correlation between $k$ and redox potential appears to exist. The general trend of the data points can be represented by curves generated from the Marcus (1968) or the Hopfield (1974) equation of vibronically-assisted tunneling (Hopfield) using reasonable values for the parameters in the equations (Figure 15). The curves shown in Figure 15 were obtained by adjusting $\nu_{ET}$ and $G^*_0$ (Marcus equation) or $r$ and $\Delta_{a+b}$ (Hopfield equation). Attempts to obtain steeper curves (which would appear to fit the data better) by nonlinear least squares analysis resulted in values for the parameters which were physically unreasonable (i.e., $\nu_{ET}$ values $10^{23}$ M$^{-1}$ s$^{-1}$ and $r < 4.5$ A). The reason for the lack of a good fit to these equations (although in part due to experimental error $\pm$ 20% and error in the fitted $k_\circ$ values $\pm$ 10%) may result from the effect of factors other than electrostatics and redox potential, e.g. steric (see below), which are not taken into consideration in the present analysis. Future determination of additional HiPIP three dimensional structures may provide the opportunity for such analysis.

A plot similar to the one shown in Figure 15 has been obtained for the reaction of a large number of $\alpha$-type cytochromes and flavodoxin semiquinone (Tollin et al. 1984), and steric arguments have been used to explain some of the deviations from the theoretical curves. Although in this case, a strong interaction mechanism (Marcus equation)
is possible, the lack of any apparent solvent exposed atoms for the [4Fe-4S] cluster in HiPIP makes a strong interaction mechanism hard to visualize for HiPIP and promotes a tunneling mechanism as an intriguing possibility. However, at present no experimental evidence exists to distinguish between the two possibilities.
Figure 15. Semilog plot of the second order rate constants extrapolated to infinite ionic strength ($k_\infty$) vs. the difference in redox potential between the reactants ($\Delta E$) for the oxidation of flavodoxin semiquinone by HiPIP

- Plot of Marcus exponential equation with $V_{ET} = 1.5 \times 10^9$ M$^{-1}$ s$^{-1}$ and $\Delta G^\ddagger(0) = 10.7$ Kcal/mole

- Plot of Hopfield tunneling equation with $R_p = 15$ Å, $r = 8.0$ Å, $N_a = 8$, $N_b = 12$, and $\Delta_{a+b} = 1.37$ ev

$R_p$ = protein radius, $r$ = the distance of closest approach between the redox centers, $N_a$ and $N_b$ are the number of atoms over which the initial and final wave functions are spread for the flavin and HiPIP, respectively, $\Delta_{a+b}$ is the vibronic coupling factor

HiPIP sources labeled as in Table 4.
Figure 15. Semilog plot of the second order rate constants extrapolated to infinite ionic strength ($k_\infty$) vs. the difference in redox potential between the reactants ($\Delta E$) for the oxidation of flavodoxin semiquinone by HiPIP.
Comparison With Free FMN Semiquinone Oxidation

Analysis of the ionic strength dependence of free FMN semiquinone oxidation by HiPIP using equations (4) and (5) indicates that the sign of the charge at the HiPIP interaction site is the same as that observed with flavodoxin oxidation (Figure 16; Table 5). This supports the idea that both reductants interact at the same site on the HiPIP surface. The magnitude of the $V_{ii}$ values for the FMN reactions are smaller for all HiPIPs than those observed for the flavodoxin semiquinone reaction. This is consistent with the larger electrostatic charge on flavodoxin.

The parameters used to obtain the $Z_1$ values in Table 5 were: $\bar{C} = 5.0$ A (an average of the values that gave the best fits for the individual HiPIPs), $Z_2 = -1.9$, $r_{12} = 8.0$ A (see above) with the $D_e$ value (31), as above, calculated from the $C. vinosum$ HiPIP/FMN data using the fitted $V_{ii}$ value and assuming $Z_1 = -1$. The fitted $V_{ii}$ values for the FMN reaction varied by $\pm 20\%$ (for different $\bar{C}$ values); thus the $Z_1$ values also have a $\pm 20\%$ error. As observed with the flavodoxin study, $C. gracile$ HiPIP gives the same $Z_1$ value as observed with $C. vinosum$. The $39\%$ smaller $Z_1$ value for $T. pfennigii$ for the FMN versus flavodoxin reaction (although just within the maximal error difference for the $Z_1$ values) can be accounted for by the smaller interaction domain for FMN, so that the FMN reaction may not be influenced by peripheral charges experienced by the larger electrostatic interaction site of flavodoxin (e.g. GLU 35). A similar argument could make for the smaller $Z_1$ values observed for FMN versus flavodoxin for $R. gelatinosa$ and the two $R. tenue$ HiPIPs.
Table 5. Effect of ionic strength on the oxidation of FMN Semiquinone HiPIP

<table>
<thead>
<tr>
<th>HiPIP Source</th>
<th>Vi (Kcal/mole)</th>
<th>(A)</th>
<th>Z1</th>
<th>k x 10^7 (M^-1 s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. vinosum</td>
<td>+2.95</td>
<td>7.4</td>
<td>-0.8 to -4.2</td>
<td>2.3</td>
</tr>
<tr>
<td>C. gracile</td>
<td>+2.87</td>
<td>7.4</td>
<td>-0.8 to -4.0</td>
<td>2.2</td>
</tr>
<tr>
<td>T. pfennigii</td>
<td>+3.24</td>
<td>7.4</td>
<td>-0.9 to -4.6</td>
<td>2.2</td>
</tr>
<tr>
<td>T. roseopersicina</td>
<td>+1.89</td>
<td>7.4</td>
<td>-0.5 to -2.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Rp. gelatinosa</td>
<td>-1.20</td>
<td>7.4</td>
<td>+0.3 to +1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Rs. tenue 3761</td>
<td>-0.79</td>
<td>7.4</td>
<td>+0.2 to +1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Rs. tenue 2761</td>
<td>-0.72</td>
<td>7.4</td>
<td>+0.2 to +1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>E. vacuolata iso-1</td>
<td>+3.78</td>
<td>7.4</td>
<td>-1.1 to -5.3</td>
<td>1.9</td>
</tr>
<tr>
<td>E. vacuolata iso-2</td>
<td>+4.08</td>
<td>7.4</td>
<td>-1.1 to -5.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\( a \) The range of \( Z_1 \) values were calculated using equation 5 and the values for given \( Z_2 = -1.9 \), with the values of other parameters as given flavodoxin reaction (see text).
The $k_\infty$ values from the fits are plotted as a function of redox potential difference in Figure 17. Although there is a substantial amount of scatter in the data, indistinguishable least squares fits can be obtained to the Marcus as well as Hopfield equations. Whether the observed deviations from the theoretical curves are significant is at present difficult to tell because of the limited number of data points and the relatively small redox potential range covered. The results are, however, again consistent with a correlation of $k_\infty$ with redox potential difference. The $\Delta G^\ddagger(0)$ value for the Marcus equation (3.7 kcal mol\(^{-1}\)) is the same as that for other HiPIP free flavosemiquinone oxidation reactions (see page 80) and much smaller than the flavodoxin oxidation value (see above). The $V_{ET}$ value (4.6 x 10\(^7\) M\(^{-1}\) s\(^{-1}\)) is smaller than that observed for the lumiflavin semiquinone reaction (9.6 x 10\(^7\) M\(^{-1}\) s\(^{-1}\)). This 48% lower value for FMN is due in part to the slightly higher pK value for the FMN semiquinone (i.e. less of the intrinsically more reactive anionic flavosemiquinone is present, page 82), and probably steric effects from the ribityl side chain (Meyer et al. 1984; page 80), and perhaps solvation effects due to the ribityl side chain and phosphate group (page 82). The decrease in $V_{ET}$ is less than that observed for the c-type cytochromes (FMN values averaged about 27% of the lumiflavin values; Meyer et al. 1984) and suggests that the phosphate group is exerting a smaller steric effect in the case of HiPIP oxidation. The $V_{ET}$ for FMN oxidation by HiPIP is also smaller than that for flavodoxin semiquinone oxidation as mentioned above, as is also true for c-type cytochromes (Tollin et al. 1984).

A striking difference between flavodoxin semiquinone oxidation
by HiPIPs (Figure 15) and cytochrome c's (Tollin et al. 1984) versus free FMN semiquinone oxidation (Figure 17; Meyer et al. 1984) is the larger dependence of the flavodoxin reaction on $\Delta E$ (which is reflected in the larger $\nu_{\rm ET}$ and $\Delta G^\ddagger(0)$ values for the fit). Expressed another way, the $k$ values for $C_\mathrm{vinosum}$ and $E_\mathrm{vacuolata}$ HiPIPs differ by ~300 fold for flavodoxin semiquinone oxidation and by ~3 fold for FMN semiquinone oxidation. The larger $\nu_{\rm ET}$ value for the flavodoxin reaction suggests that placing a flavosemiquinone (FMN in the present case) into a protein environment increases the intrinsic reactivity of the flavin (Tollin et al. 1984). The larger value for $\Delta G^\ddagger(0)$ for the flavodoxin reaction, as compared to free FMN (see below), suggests more structural rearrangements are required to reach the transition state for flavodoxin semiquinone versus free FMN semiquinone (Tollin et al. 1984).

The effects of electrostatics is, as already mentioned, larger for flavodoxin than for FMN. For example, the rate constant value increases ~9 fold for flavodoxin semiquinone oxidation by $C_\mathrm{vinosum}$ HiPIP versus ~3 fold for FMN semiquinone oxidation over an ionic strength range of 0.014-0.49 M (the 0.014 M rate constant for FMN semiquinone oxidation was calculated from the parameters in Table 6). This compares with the rate constants for tuna cytochrome c oxidation of FMN and flavodoxin semiquinones which vary by a factor of 3 and 100 over an ionic strength range of 0.058-0.5 M. The larger effect of ionic strength with cytochrome c is consistent with the larger electrostatic field near the proposed site of electron transfer (the exposed heme edge, cf. Tollin et al. 1984; Tollin 1984, personal
The large number of available cytochrome c three dimensional structures has allowed for structural interpretations of deviations observed from k versus E plots. Such analysis has revealed steric hindrance at the active site among equal redox potential cytochromes as the reason for the differences in reactivity with both free FMN and flavodoxin semiquinones (Meyer et al. 1984; Tollin et al. 1984). At present, the lack of such structural information on HiPIPs precludes a similar analysis of the data in Figure 15.

The larger magnitude of the steric effects, as well as the redox potential and electrostatic effects, observed for flavodoxin semiquinone oxidation by cytochromes versus that for free FMN have resulted in the suggestion that these factors may be involved in the determination of the biological specificity of electron transfer (Tollin et al. 1984). A similar conclusion can be made from the present study with regards to electrostatics and redox potential effects.
Figure 16. Plots of the apparent second order rate constants for FMN semiquinone oxidation by HiPIP versus the square root of the ionic strength.

Curves represent calculated theoretical values for the data obtained using equation (4) see text for details. Curves for different HiPIPs labeled as in Table 4.
Figure 17. Semilog plot of the apparent second order rate constants extrapolated to infinite ionic strength ($k_\infty$) vs. the difference in redox potential between the reactants ($\Delta E$) for FMN semiquinone oxidation by HiPIP.

--- plot of Marcus exponential equation with $\gamma_{ET} = 4.6 \times 10^7$ M$^{-1}$ and $\Delta G^+(0) = 3.7$ Kcal/mole

--- plot of Hopfield tunneling equation with $r = 7.7$ A and $\Delta_{a+David} = -.66$ eV, all other values as given in Figure 15 as are definition of parameters. HiPIP sources labeled as in Table 4.
Conclusions

Electrostatically correcting (to infinite ionic strength) the rate constants for free and protein-bound FMN semiquinone oxidation by HiPIP allows for the examination of the influence of redox potential difference on the rate constant for oxidation. The potential dependence of $k$ for flavodoxin oxidation is much larger in magnitude than that for free FMN ($\sim 300$ fold vs. $\sim 3$ fold over a $210 \text{ mV}$ potential range). This compares to the same reactions involving $c$-type cytochromes, where $k$ varied by approximately a factor of 100 and 5 over a $0.18-0.5 \ E_m$ potential range for FMN and flavodoxin semiquinone oxidation, respectively. Analysis of the $k$ versus $E$ data using the Marcus exponential equation suggests that placing FMN semiquinone into a protein environment increases the intrinsic reactivity of the flavin and the structural rearrangements required to reach the transition state.

As expected electrostatic effects are also larger for flavodoxin versus free FMN. The rate constant increases by a factor $\sim 9$ for the flavodoxin reaction with $C. \ vinosum$ HiPIP and $\sim 3$ fold for the FMN reaction over an ionic strength range $0.014-0.49 \text{ M}$. This compares with cytochrome $c$ which varies by a factor of $\sim 100$ and by a factor $\sim 3$ for the same reactions over an ionic strength range of $0.058-0.5 \text{ M}$. Calculated charges at the electrostatic interaction site on HiPIP with both FMN and flavodoxin semiquinones vary with expectation based on charged residue substitutions at a localized region of the protein (where the cluster comes closest to the protein surface) previously suggested to be involved in iron hexacyanide electron transfer (Mizrahi
et al. 1980).

The results are similar in many respects to those observed in the reaction of flavodoxin and FMN semiquinones with g-type cytochromes (Tollin et al. 1984). On the basis of both the HiPIP and cytochrome results it appears that the effects of electrostatics, sterics, and redox potential are much larger for protein-bound flavosemiquinones than free, independent of the oxidant chromophore type or its extent of solvent exposure. Thus, the factors of electrostatics, sterics, and redox potential can form the basis of specificity in biological protein-protein electron transfer reactions.
KINETICS OF REDUCTION OF Clostridium pasteurianum RUBREDOXIN BY LASER PHOTOREDUCTED SPINACH FERREDOXIN:NADP⁺-REDUCTASE AND FREE FLAVINS

At low ionic strength (I = 10 mM), C. pasteurianum Rdox gives a maximal visible difference spectrum for complex formation at an [Rdox]/[FNRox] ratio of 1:1 (Figure 18 and inset). The spectrum is very similar to that seen with M. elsdenii Rdox and FNRox (Foust et al. 1969), although there are some small, but distinct differences between the spectra. The C. pasteurianum Rdox:FNRox is apparently dissociated at high ionic strength (I=310 mM), as suggested by the loss of the difference spectrum at high ionic strength. Sedimentation equilibrium experiments have shown the M. elsdenii Rdox:FNRox to be dissociated at high ionic strength. Complex formation also produces a difference CD spectrum (Figure 19). Due to the overlapping spectral transitions of Rdox and FNRox in the wavelength region of the difference spectra, no definitive assignment can be made concerning the origin of the spectra.

Free Flavosemiquinone Reduction of Uncomplexed Redox Protein

Kinetic experiments were performed under pseudo first order conditions in which the concentrations of oxidized redox proteins was in large excess over that of the free flavosemiquinone (or FNR semiquinone) generated by the laser flash (≈ 0.1 μM/flash). The kinetics of reduction of free Rdox and FNRox by photoproduced free flavin semiquinonones exhibit no complexities. Figure 20a presents a typical reaction trace for a solution containing oxidized
Figure 18. Absorption difference spectrum for \( \text{R}_{\text{dox}}:\text{FNR}_{\text{oxy}} \) complex. Difference spectrum shown is the maximum obtained; inset shows titration to this maximal spectrum. The \( \Delta \varepsilon_{390\ \text{nm}} \) has the units of M \( \cdot \) cm\(^{-1}\). Concentrations and absorbances were corrected for dilution. Final concentrations of \( \text{Rd}_{\text{ox}} \) and \( \text{FNR}_{\text{oxy}} \) were 138 \( \mu \text{M} \) and 108 \( \mu \text{M} \), respectively. Experiments were performed in 4 \( \text{mM} \) potassium phosphate, 0.5 \( \text{mM} \) EDTA, pH 7, \( I = 10 \text{ mM buffer} \).
Figure 19. Difference CD of RDox:FNRox complex.

a) C. pasteurianum Rdox, pH 7, I = 10 mM. b) Spinach FNRox pH 7, I = 10 mM. c) 1:3:1 mixture of Rdox/FNRox pH 7, I = 10 mM. d) Same ratio and concentrations of proteins as in c) but unmixed. e) Difference (c) - (d). f) Equimolar Rdox/FNRox mixture pH 7, I = 10 mM. g) (f) + 0.3 M KCl pH 7, I = 310 mM. h) difference (f) - (g).
Figure 19. Difference CD for $\text{RD}_{\text{ox}}:\text{FNR}_{\text{ox}}$ complex.
5-deazariboflavin (5-dRf*). Upon laser flash photolysis, a rapid absorbance increase occurs due to the formation of 5-dRf semiquinone (5-dRf·) through excited triplet state quenching via electron transfer from EDTA. The subsequent rapid second order decay of 5-dRf· (Figure 20a insert) is due to the disproportionation reaction (Edmondson et al. 1972). Addition of Rdox to the 5-deazariboflavin solution results in a more rapid reoxidation of 5-dRf* and also leads to a net decrease in absorbance (bleach) due to Rd ox reduction (Figure 20b). Semilog plots of the 5-dRf* decay and Rd ox (Figure 21b insert) were linear through 3-4 half-lives and over an approximately 10-fold Rd ox concentration range, consistent with pseudo first order kinetics. A plot of the dependence of kobs on Rd ox concentration was linear (Figure 27a) with an apparent second order rate constant for Rd ox reduction by 5-dRF* of 3.0 x 10^8 M^-1 s^-1 (I = 10 mM, pH 7). It should be mentioned that in addition to the 5-dRF* reaction, a much slower second order reduction of Rd ox was also observed (data not shown; k ~ 10^7 M^-1 s^-1, although the kinetics may have more than one component). Though this may be at least partly due to fully-reduced 5-deazariboflavin, we were unable to measure any reduction by the steady-state generated light/EDTA/5-deazariboflavin photoproduct. However, it has been suggested that this latter product is not identical with authentic fully-reduced 5-deazariboflavin (Massey and Hemmerich 1978). Since identification of the reducing species involved in this slow process would be difficult, it was not further investigated.

Laser-induced reduction of FNR ox can be monitored by following 5-dRf* oxidation at 504 nm, an FNR oxidized/semiquinone isosbestic
wavelength or at 590 nm (Figure 20c) where the protein-bound neutral FAD semiquinone absorbs. Pseudo first order rate constants were the same, within experimental error, at the two wavelengths. Plots of $k_{obs}$ vs. FNR$_{ox}$ concentration for the data obtained at 504 nm were linear over the concentration range studied with an apparent second order rate constant of $5.5 \times 10^8$ M$^{-1}$ s$^{-1}$ (Figure 27b). As with the Rd$_{ox}$/5-dRf reaction, a much slower transient decay was observed (data not shown).

As expected on thermodynamic grounds, lumiflavin semiquinone (LfH$^\cdot$) was not measurably reactive with FNR$_{ox}$ (up to 50 $\gamma$M protein; data not shown). We have therefore used this flavin to investigate the relative reactivities of Rd$_{ox}$ free and bound to FNR$_{ox}$. The results establish that Rd$_{ox}$ is reducible by LfH$^\cdot$ when complexed with no detectable alteration in the rate constant for reduction ($k = 1.3 \times 10^7$ M$^{-1}$s$^{-1}$; Figure 22). The much larger second order rate constant for Rd$_{ox}$ reduction by 5-dRf$^\cdot$ vs. LfH$^\cdot$ is consistent with the lower redox potential for 5-dRf$^\cdot$ (-650 mV vs. -231 mV, respectively; Draper and Ingraham 1968, Blankenhorn 1976).

**Intracomplex Oxidation of FNR Semiquinone by Rdox**

Based on the relative redox potentials of Rd (-57 mV; Lovenberg and Sobel 1965) and the FNR oxidized/semiquinone redox couple (-320 mV; Keirns and Wang 1972; however see also Batie and Kamin 1981 and Smith et al. 1981) it is expected that an intracomplex electron transfer reaction would involve irreversible electron transfer from FNR semiquinone to Rd$_{ox}$. To investigate the possible occurrence of this reaction, we added increasing amounts of a solution containing equal
concentrations of Rdox and FNRox to a low ionic strength solution (I = 10 mM) containing 5-deazariboflavin. Under these conditions, most of the added protein is complexed and any complexed FNR semiquinone which is produced would be expected to transfer its electron intramolecularly to Rdox. By analogy to the LfH reaction, direct reduction of complexed Rdox by 5d-Rf should also be possible (see below), but this will be a second order process and thus kinetically distinguishable from an intracomplex reaction. The absorbance changes (relative to the preflash baseline) expected for the various reacting species are as follows. For the second-order direct 5-d-Rf reaction with the complex, one-electron reduction of FNRox will result in an increase in absorbance at 590 nm and no absorbance change at 504 nm, the FNRox/FNR isobestic wavelength. For direct 5-d-Rf reduction of complexed Rdox an absorbance decrease would be expected at both 590 and 504 nm. For intracomplex electron transfer from FNR to Rdox an absorbance decrease would be expected with components above and below the baseline. At 590 nm, the mixture, upon laser photolysis, gives a first order decay (Figure 23c) which is different from the decay kinetics obtained with solutions containing equivalent concentrations of the individual proteins (Figures 23a and 23b). The observed first order rate constant, however, does not increase linearly with increasing amounts of the mixture, but instead remains constant (k = 2.0 x 10^3 s⁻¹), within an experimental uncertainty of ± 15%, after the first few additions (Figure 23c insert). Thus, the observed transient must reflect a true first order reaction rather than a pseudo first order reaction. We attribute the slightly smaller kobs values at the lowest concentrations...
Figure 20. Reduction of Rd\textsubscript{ox} and FNR\textsubscript{ox} by photoproduced 5-deazariboflavin semiquinone.

a) Formation and second order decay of 5-dRf\textsuperscript{•} (disproportionation) at 504 nm under anaerobic conditions in 4 mM potassium phosphate, 0.5 mM EDTA buffer, pH 7 and I = 10 mM, 89 um 5-deazariboflavin. b) Transient decay curve obtained at 504 nm on addition of 25 uM Rd\textsubscript{ox}\textsuperscript{'} solution conditions as in a). c) Transient absorbance change observed at 590 nm upon addition of 13 uM FNR\textsubscript{ox}\textsuperscript{'} solution conditions as in a). Insert a) is a second order plot of 5-dRf\textsuperscript{•} disproportionation (baseline used for the end of the reaction was the preflash baseline). Inserts b) and c) are semilog plots for the corresponding transient decay curves shown.
Figure 20. Reduction of $R_{d\text{ox}}$ and $P_{N\text{ox}}$ by photoproduced 5-deazariboflavin semiquinone.
Figure 21. Dependence of $k_{\text{obs}}$ for 5-dRf decay upon a) $Rd_{\text{ox}}$ b) $\text{FNR}_{\text{ox}}$ concentrations.

Both reactions were monitored at 504 nm. Reactions were performed using 4 mM potassium phosphate, 0.5 mM EDTA, pH 7 and $I = 10$ mM and 89 uM 5-deasariboflavin under anaerobic conditions.
Figure 22. Dependence of $k_{obs}$ for LfH$^*$ decay upon $R_{d_{ox}}$ concentration (o) and mixtures containing equal concentrations of $R_{d_{ox}}$ and FNR$_{ox}$ (x). Concentrations for both reactions expressed as the concentration of $R_{d_{ox}}$ added. Lumiflavin (50 uM) was in 4 mM potassium phosphate, 0.5 mM EDTA buffer pH 7 and $I = 10$ mM.
to the occurrence of parallel second order reactions involving free
\( \text{FNR}_\text{ox} \) (and \( \text{Rd}_\text{ox} \)) present in equilibrium with the complex.

The transient shown in Figure 23c has components both above and
below the preflash baseline. The ratio of the absorbance above the
preflash baseline (obtained by extrapolating the decay curve to \( t = 0 \))
to that below the preflash baseline (1.2) is in reasonable agreement
with the ratio of \( \Delta \varepsilon_{\text{FNR}^\cdot/\text{FNR}_\text{ox}} \) to \( \Delta \varepsilon_{\text{Rd}_\text{ox}/\text{Rd}_{\text{red}}} \) (1.1). Similar
quantitative agreement is also obtained for the transients shown in
Figures 25a and 26b (see below). The first order process of Figure 23c
therefore can be assigned to intracomplex electron transfer from FNR
semiquinone to \( \text{Rd}_\text{ox} \). The flash-induced difference spectrum obtained
for this process supports this interpretation (Figure 23). Specifically, the absorbing species immediately following the laser
flash has spectral properties consistent with the \( \text{FNR}^\cdot \) minus \( \text{FNR}_\text{ox} \)
steady-state difference spectrum (Figure 23). Furthermore, the total
decrease in absorbance (i.e. the amplitude of the transient signal
below the preflash baseline) is similar in shape to the \( \text{Rd}_\text{ox} \) minus
\( \text{Rd}_{\text{red}} \) steady-state difference spectrum. The small differences between
the flash data and the steady state spectra are within our experimental
uncertainty.

At 590 nm, the increase in absorbance due to \( \text{FNR}_\text{ox} \) reduction by
5-dRf (cf. Figure 23a) causes cancellation (cf. Figure 23c) of the
decrease in absorbance (cf. Figure 23b) due to any direct \( \text{Rd}_\text{ox} \)
reduction by 5-dRf. However, as Figure 23d shows, at 504 nm, which is
an \( \text{FNR}_\text{ox}/\text{FNR}^\cdot \) isosbestic wavelength, rapid 5-dRf oxidation reactions
can be seen, i.e. biphasic kinetics are observed. As expected,
Figure 23. Laser-flash-induced kinetic properties of mixtures containing equal concentrations of $Rd_{ox}$ and $FNR_{ox}$.

Transient absorbance changes at 590 nm were measured upon laser photolysis of solutions which were 89 $\mu$M in 5-deazariboflavin (pH 7 and $I = 10$ mM) and a) 23.1 $\mu$M in $FNR_{ox}$, b) 23.1 $\mu$M in $Rd_{ox}$, or c) a mixture 23.1 $\mu$M in $Rd_{ox}$ and 23.1 $\mu$M in $FNR_{ox}$ in the presence of 89 $\mu$M 5-deazariboflavin (pH 7 and $I = 10$ mM) under anaerobic conditions. Transient absorbance change at 504 nm d) were measured with a solution 23.1 $\mu$M in $Rd_{ox}$ and 23.1 $\mu$M in $FNR_{ox}$, other conditions above. Insert c), dependence of $k_{obs}$ for the transient observed at 590 nm (X) and the slow phase transient (see text for discussion) at 504 nm (●,●) on the total concentration of $Rd_{ox}$ (or $FNR_{ox}$) added from a concentrated stock solution of an equal concentration mixture of $rd_{ox}$ and $FNR_{ox}$.
Figure 23. Laser-flash-induced kinetic properties of mixtures containing equal concentrations of RdOX and FNRox.
Figure 24. Flash-induced difference spectra associated with the first order reaction of FNR:Rd complex.

Laser flash data were obtained with a sample 25 μM in both FNROx and RDox and 85 μM in 5-deazariboflavin. The buffer was 4 mM potassium phosphate, 0.5 mM EDTA, pH 7 and I = 10 mM. The flash data indicated by (X) represent the signal amplitude below the preflash baseline at each wavelength. The data represented by (0) were obtained by extrapolating the first order portion of the reaction to zero time and subtracting from this value the values given by (X) in order to obtain the spectrum of the species initially formed by the reaction with 5-dRF. The steady-state FNR* minus FNROx difference spectrum (---) was obtained by phototitration using 5-deazariboflavin/EDTA as the reducing system. The steady-state RDox minus RDred spectrum (----) is that of RDox; reduced Rd has no appreciable absorbance in the wavelength region of the difference spectrum (see Lovenberg and Sobel 1965). The flash data were normalized to the steady-state spectra at 590 nm and 490 nm for the data indicated by (0) and (X), respectively.
Figure 24. Flash-induced difference spectra associated with the first order reaction of FNR:Rd complex.
however, the slow component of the 504 nm transient has the same rate constant and the same lack of concentration dependence as does the transient observed at 590 nm (Figure 23c insert). In fact, within experimental error, the rate constant for this reaction is independent of wavelength from 490-610 nm.

Increasing the ionic strength results in the disappearance of the first order transient and the appearance of a decay curve which displays complex kinetics (Figure 25). Thus, the effect of ionic strength is qualitatively consistent with the dissociation of the M. elsdenii Rd<sub>ox</sub>:FNR<sub>ox</sub> complex at high ionic strength (Foust et al. 1969), as well as with our own observations with the C. pasteurianum Rd<sub>ox</sub> complex (see above).

**Bimolecular Oxidation of FNR Semiquinone by Rd<sub>ox</sub>**

In order to measure the second order reaction between FNR semiquinone and Rd<sub>ox</sub>, we have carried out flash experiments with excess FNR<sub>ox</sub> in the presence of increasing amounts of Rd<sub>ox</sub>. Upon addition of a substoichiometric amount of Rd<sub>ox</sub> to a FNR<sub>ox</sub>-containing sample at low ionic strength (I = 10 mM), the otherwise kinetically stable FNR semiquinone (Figure 26a) is found to undergo a first order decay, superimposed upon a bleach due to Rd<sub>ox</sub> reduction (Figure 26b). Under these experimental conditions, the majority of the FNR is free whereas essentially all of the Rd<sub>ox</sub> is complexed (see above). Thus, the transient represents predominately electron transfer between free FNR semiquinone and complexed Rd<sub>ox</sub>. This would be consistent with the fact that complexation still allows facile reduction of Rd<sub>ox</sub> by LfH<sup>+</sup> and 5-
Figure 25. Transient absorbance change at 590 nm upon laser flash excitation of a solution containing 13 uM or Rd_{ox} and of FNR_{ox} in the presence of 89 uM 5-deazariboflavin at pH 7 and a) I = 10 mM or b) I = 310 mM.
Figure 26. Laser-flash-induced kinetic properties of solutions containing a fixed [FNRox] and increasing amounts of Rdox.

Transient absorbance changes at 590 nm upon laser flash photolysis of solutions a) 35 μM in FNRox, b) a mixture 2.5 μM in Rdox and 34.8 μM in FNRox, [Rdox]/[FNRox] = 0.07. Insert (b), dependence of kobs for the 590 nm transient on the [Rdox]/[FNRox] ratio. The concentration of FNRox was corrected for dilution. The line drawn through the lower [Rdox]/[FNRox] points of insert (b) yields a second order rate constant of 5.0 x 10^7 M^-1 s^-1. The horizontal line represents the kobs value (2.0 x 10^3 s^-1) obtained from the experiment described in Figure 24.
Figure 26. Laser-flash-induced kinetic properties of solutions containing a fixed $[FNR_{ox}]$ and increasing amounts of $Rd_{ox}$. 
dRf⁺ (see above). The possibility exists that FNR⁺ also reacts with free Rd ox (in equilibrium with the complex). Assuming that FNR⁺ only reacts with free Rd ox and that the Kd for the *M. elsdenii* Rd ox:FNROx complex (Foust et al. 1969) is applicable to the present system, then free Rd ox would need to be ~ 600 fold more reactive towards FNR⁺ at I = 10 mM than at I = 310 mM (see below).

As expected for a second order process, the observed rate constant for the reaction shown in Figure 26b increases with increasing amounts of added Rd ox⁺. As the [Rd ox]/[FNROx] ratio approaches unity, where the majority of FNROx is complexed, the kobs value begins to level off (Figure 26b insert). The rate constant for the second order portion of the reaction is $5 \times 10^7$ M⁻¹s⁻¹. This is not an unreasonable value for a facile reaction between two macromolecules with a large redox potential difference, i.e. it is probably close to being diffusion-controlled. As would be expected, the limiting first order rate constant (see Figure 26) is approximately the same as that found in the experiment described in Figure 23. The kinetics observed under these conditions at 504 nm are biphasic at all [Rd]/[FNR] ratios (the fast phase again being due to the direct reactions with 5-dRf⁺), with the slow phase giving identical results to those obtained at 590 nm (data not shown).

The reaction of free FNR semiquinone with free Rd ox⁺ can be measured at high ionic strength in a similar experiment. The apparent second order rate constant is $4 \times 10^6$ M⁻¹s⁻¹ (I = 310 mM). The decrease in rate constant at high ionic strength may in part be due to electrostatic effects involving localized opposite charges on the
Conclusions

Little information is currently available on intracomplex electron transfer processes between redox proteins (summarized in Table 6). It is interesting that the flavodoxin/cytochrome c system possesses a larger driving force than the Rd/FNR system and, based on putative complexes generated by computer modeling, has portions of the heme and flavin chromophores within Van der Waals contact distance, and yet a relatively small first order rate constant is found (Simonsen et al. 1982). Comparison of the intracomplex and intramolecular electron transfer systems shown in Table 6 indicates no absolute correlation between thermodynamic driving force and first order rate constants, although this relationship may exist to a limited extent (e.g. spinach ferredoxin/FNR vs. rubredoxin/FNR). This is different from what is found for the second order reduction of homologous redox proteins containing heme c or [4Fe-4S] prosthetic groups by photoreduced free flavins (Meyer et al. 1983) and suggests that factors other than redox potential (e.g. relative orientation of redox cofactors, structural reorganizations) may also be important in determining intracomplex and intramolecular electron transfer. The exact nature of such effects is not known in detail as yet, but in a multiheme-containing cytochrome from Pseudomonas aeruginosa, cytochrome cd1, the perpendicular orientation between hemes c and d1 has been suggested to account for the slow intramolecular electron transfer observed between them.
Table 6. First order rate constants for intracomplex or intramolecular electron transfer in various redox systems.

<table>
<thead>
<tr>
<th>TYPE OF ELECTRON TRANSFER: Proteins</th>
<th>Redox Centers/ΔE(V)</th>
<th>F Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INTRACOMPLEX</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em> flavodoxin/horse heart cytochrome c</td>
<td>FMNH\textsuperscript{−}→heme c Fe\textsuperscript{3+}/0.402\textsuperscript{2}</td>
<td></td>
</tr>
<tr>
<td>Bovine adrenal NADPH: adrenodoxin reductase/bovine adrenal adrenodoxin</td>
<td>FADH\textsuperscript{−}→[2Fe-2S]\textsuperscript{2+}/0.04\textsuperscript{3}</td>
<td></td>
</tr>
<tr>
<td>Spinach FNR/Spinach ferredoxin</td>
<td>FADH\textsuperscript{−}→[2Fe-2S]\textsuperscript{2+}/-0.16\textsuperscript{2}</td>
<td></td>
</tr>
<tr>
<td>Spinach FNR/ <em>Clostridium pasteurianum</em> Rd</td>
<td>FADH\textsuperscript{−}→Fe\textsuperscript{3+}/0.263</td>
<td></td>
</tr>
<tr>
<td><strong>INTRAMOLECULAR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorobium thiosulfatophilum</em> flavocytochrome c</td>
<td>8-κ-cysteiny1→heme c Fe\textsuperscript{3+}/0.06</td>
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</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> cytochrome cd\textsubscript{1}</td>
<td>heme c Fe\textsuperscript{2+}→heme cd\textsubscript{1} Fe\textsuperscript{3+}/0.007</td>
<td></td>
</tr>
<tr>
<td><em>Hyphomicrobi um X</em> trimethylamine dehydrogenase</td>
<td>6-S-cysteiny1-FMNH\textsubscript{2}→[4Fe-4S]\textsuperscript{2+}/not known</td>
<td></td>
</tr>
<tr>
<td><em>Hansenula anomala</em> flavocytochrome d\textsubscript{2}</td>
<td>FMNH\textsubscript{2}→heme d\textsubscript{2} Fe\textsuperscript{3+}/0.016</td>
<td></td>
</tr>
<tr>
<td>Milk xanthine oxidase</td>
<td>Mo(V)→[2Fe-2S]\textsubscript{I}\textsuperscript{2+}/0.008\textsuperscript{4}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mo(V)→[2Fe-2S]\textsubscript{II}\textsuperscript{2+}/0.096\textsuperscript{4}</td>
<td></td>
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<tr>
<td></td>
<td>Mo(V)→[2Fe-2S]\textsubscript{II}\textsuperscript{2+}/0.056\textsuperscript{4}</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1}Unless indicated otherwise, redox potentials used to calculate were those cited in reference given.

\textsuperscript{2}Meyer et al. 1983.

\textsuperscript{3}Kamin et al. 1980.

\textsuperscript{4}Porras and Palmer 1982.
<table>
<thead>
<tr>
<th>First Order Rate Constant (s(^{-1}))</th>
<th>Comments</th>
<th>References</th>
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<tr>
<td>22</td>
<td>85</td>
<td>Ionic strength dependent</td>
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<tr>
<td></td>
<td>1.9</td>
<td>NADPH bound to flavoprotein</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>NADP+ bound to flavoprotein</td>
</tr>
<tr>
<td></td>
<td>2100</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td><em>not known</em></td>
<td>23</td>
<td>Binding of substrate to FMNH(_2) form decreases rate constant</td>
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<td>380</td>
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<tr>
<td></td>
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<tr>
<td>4</td>
<td>11.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.8</td>
<td></td>
</tr>
</tbody>
</table>

*were those cited*
(Makinen et al. 1983; cf. Table 6). The possibility of redox enzymes altering structural factors in order to control the rates of intermolecular or intramolecular electron transfer is intriguing. In this regard, the observation that the rate constant for intramolecular electron transfer is modified in the redox enzyme, trimethylamine dehydrogenase, by conformational changes induced by substrate binding to the flavohydroquinone form of the enzyme is of considerable interest (Steenkamp and Beinert 1982; Table 6).

Several lines of evidence have emphasized the importance of complementary charged residue interactions in stabilization of redox protein complexes (Foust et al. 1969, Salemme 1976, Poulos and Kraut 1980, Smith et al. 1980, Mauk et al. 1982, Simonsen et al. 1982, Waldmeyer et al. 1982, Chan et al. 1983, Matthew et al. 1983, Poulos and Mauk, 1983). The present kinetic data are unable to distinguish between the large number of charged side-chains spread across a surface of the protein away from the Fe center (Watenpaugh et al. 1979) and a ring of negative charges (residues 14, 19, 36, 48, and 50) in closer proximity to the Fe center (Adman 1979) as possible FNR recognition sites. However, additional kinetic studies with other Rd s of known sequence may help do so. Further, structural characterization of the complex by such techniques as NMR or by computer modeling (upon completion of the FNR structure, see Karplus and Herriott 1982) may better define possible recognition sites.
HIPIP CIRCULAR DICHROISM

The four spectral groups of the comparative HiPIP CD survey presented below are based on the reduced visible (320-600 nm) spectra. The division of the spectra into groups is based on the similarity of the spectral properties as well as spatial economy and clarity of presentation. The visible wavelength range (versus the near or far UV) was chosen since only the [4Fe-4S] cluster absorbs in this region and thus the spectra should be the most similar to each other. Carter's prediction (1977b; see page 9) concerning a correlation between CD and HiPIP redox behavior, which this work examines, is based on the reduced (i.e., [4Fe-4S]^{2+} cluster oxidation level) visible CD and thus this oxidation state was used for the spectral grouping.
Visible Reduced CD Spectra

Group A (Figure 27) contains: *C. vinosum*, *C. gracile*, and *T. roseopersicina* HiPIPs. The similarity of their spectra is consistent with the high degree of sequence homology between these HiPIPs (Table 1). Although group B, which contains *Paracoccus sp.* and the two iso-HiPIPs from *E. vacuolata*, could have, on qualitative grounds, been placed in group A, this was not done for sake of clarity of presentation. In addition, this group in general has a lower $E_{m,7}$ values than those of group A and are from moderately halophilic bacteria. Group C contains the net positively charged HiPIPs (based on sequence data) *Rr. gelatinosa* HiPIP and the HiPIPs from two strains of *Rs. tenue* (3761 and 2761). The similarity in the two *Rs. tenue* HiPIP spectra again is consistent with the high degree of sequence homology between the HiPIPs (Table 1). The group C spectra, in some respects, qualitatively resemble those of group A, however, the presence of positive ellipticity at $\lambda=330$ nm warranted their placement into a separate group. Group D contains *T. pfennigii* HiPIP, *Rp. globiformis* HiPIP, and the two iso-HiPIPs from *E. halophila*. These HiPIPs display the most divergent spectra from those of group A. The two iso-HiPIPs from *E. halophila*, as with those from *E. vacuolata*, exhibit similar spectra. Although group D could be subdivided into additional groups, this was not done.
Figure 27. Reduced visible HiPIP CD spectra at pH 7.

Group A: C. vinosum (---), C. gracile (-- --), T. roseopersicina (••••); Group B: Paracoccus sp. (---), E. vacuolata iso-1 (-- --), E. vacuolata iso-2 (--•--•--•--); Group C: Rps. gelatinosa (---), Rs. tenue 3761 (-- --), Rs. tenue 2761 (-- --); Group D: T. pfennigii (••••), Rps. globiformis (-- •--), E. halophila iso-1 (-- --), E. halophila iso-2 (-- --)
The ellipticity values associated with the [4Fe-4S] cluster electronic transitions in the various HiPIPs and other [4Fe-4S]-containing ferredoxins are in general quite small in comparison to the oxidized chromophores of [2Fe-2S] ferredoxins and rubredoxins (cf. Stephens et al. 1978 and references cited therein). This fact, coupled with the small uncertainty (estimated ± 25%) in the HiPIP extinction coefficients, may lead to errors in the magnitudes for the spectra and therefore, at present, only major qualitative differences are of significance.

As noted previously (Flatmark and Dus 1969) the single unresolved maximum (375-388 nm; Bartsch 1978, see Figure 3) in the 350-600 nm region of the absorption spectrum is, based on the reduced visible CD, composed of 5-6 transitions (C. vinosum HiPIP: negative maxima at ~347, 394, and 535 nm; positive maxima at ~456 and 590 nm with a positive shoulder at ~500 nm). At present, the description of the electronic structure for the [4Fe-4S]^{2+} cluster is only qualitative in nature. The electronic origin for the major cluster visible absorption band, as well as the major cluster band occurring in the near UV (see below), has been reported to arise from primarily S --- Fe charge transfer transitions (cf. Aizman and Case 1982). The results of the present study may aide in the refinement of the presently available description of the cluster electronic structure.

The geometrical cluster properties for only a single reduced HiPIP, C. vinosum, have been determined (cf. Carter 1977b, and page 16). The observation that isoelectronic [4Fe-4S] clusters in two unrelated ferredoxins exhibit essentially identical structures to that
of HiPIP (cf. Stout 1982 and references cited therein), however, suggests that the reduced cluster geometry in various HiPIPs is probably conserved. Thus, differences observed among the visible HiPIP CD spectra (Figure 27) are most likely due to perturbations of the cluster transitions by polypeptide interactions. Carter (1977b) has taken this argument a step further and suggested that the clusters are prochiral and that the observed optical activity in the visible wavelength region is induced by binding the cluster in an asymmetric protein environment.

The correlation of visible CD and redox behavior proposed by Carter (1977b) is based, in part, on features of the native reduced _C. vinosum_ HiPIP spectrum and that in 85% DMSO (pH 9.85). Specifically, the presence and magnitude of the two negative bands at 348 and 394 nm, possibly arising from TYR-19 interactions with the cluster, were suggested to be correlated with HiPIP redox properties (Carter 1977b, see also page 9). The visible CD spectrum observed for reduced _C. vinosum_ HiPIP (Figure 27 A) is in good agreement with earlier reports (Flatmark and Dus 1969; Hall et al. 1974; Stephens et al. 1978). A comparison of reduced spectra for other HiPIPs (Figure 27), indicates that negative maxima at approximately 350 and 400 nm are conserved in many, but not all HiPIPs. Spectra for the HiPIPs in group D (Figure 27) indicate a dramatic alteration in these maxima, particularly the one at ~400 nm. Oxidized _E. halophila_ iso-2-HiPIP, the HiPIP with lowest redox potential observed to date (Table 7) has been found to give an EPR spectrum (Cammack 1979) identical to other HiPIPs (Antanaitis and Moss 1975; Blum et al. 1978; Sands 1979; Zorin and
Gogotov 1984), yet this protein (as well as iso-l-HiPIP from the same bacterium) exhibits a dramatically altered visible CD from that of C. vinosum. Thus, a strict correlation between utilization of the 3+,2+ [4Fe-4S] cluster oxidation levels and visible CD is not observed. It is interesting that certain spectral features of the E. halophila iso-HiPIP spectra appear to exhibit an enantiomeric relationship to features in the C. vinosum spectrum. This point is discussed in more detail below. *Rp. globiformis* HiPIP, which has the highest redox potential of any HiPIP to date (Table 7), and the *E. vacuolata* iso-HiPIPs (as compared to *C. vinosum* HiPIP) have spectra that indicate no strict, simple correlation exists between the magnitude of the negative maxima at ~ 350 nm and ~ 400 nm and redox potentials of proteins using the 3+,2+ [4Fe-4S] cluster oxidation levels. Based on the present alignment of amino acid sequence data, *E. halophila* iso-HiPIPs and *T. pfennigii* HiPIP appear to have TYR-19 conserved (Table 1). Thus, it appears that no simple relationship exists between the negative maxima and the presence of TYR-19. Whether or not TYR-19 occupies a structurally similar position with respect to the cluster, as found in *C. vinosum* HiPIP, awaits verification by X-ray crystallographic studies.
Table 7. HiPIP redox potentials.

<table>
<thead>
<tr>
<th>HiPIP Source</th>
<th>$E_{m,7}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rp. globiformis</em></td>
<td>+453</td>
</tr>
<tr>
<td><em>C. vinosum</em></td>
<td>+356$^a$</td>
</tr>
<tr>
<td><em>T. pfennigii</em></td>
<td>+352$^a$</td>
</tr>
<tr>
<td><em>C. gracile</em></td>
<td>+347</td>
</tr>
<tr>
<td><em>T. roseopersicina</em></td>
<td>+342 (+294)$^c$</td>
</tr>
<tr>
<td><em>Rp. gelatinosa</em></td>
<td>+332$^a$</td>
</tr>
<tr>
<td><em>Rs. tenue</em> 3761</td>
<td>+304</td>
</tr>
<tr>
<td><em>Rs. tenue</em> 2761</td>
<td>+302</td>
</tr>
<tr>
<td><em>Paracoccus sp.</em></td>
<td>+282$^a$</td>
</tr>
<tr>
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<td>+260</td>
</tr>
<tr>
<td><em>E. vacuolata</em> iso-2</td>
<td>+150</td>
</tr>
<tr>
<td><em>E. halophila</em> iso-1</td>
<td>+110</td>
</tr>
<tr>
<td><em>E. halophila</em> iso-2</td>
<td>+50</td>
</tr>
</tbody>
</table>

$^a$ from Mizrahi et al. 1980

$^b$ $E_m$ value at pH 9.0

$^c$ $E_m$ value at pH 8.7
Visible Oxidized CD Spectra

The oxidized CD spectra of HiPIP are shown in Figure 28. The spectrum for *C. vinosum* HiPIP differs from the three previously published (Flatmark and Dus 1969; Hall et al. 1974; and Stephens et al. 1978). The spectrum of Flatmark and Dus (1969) has been suggested by Stephens et al. (1978) to be incorrect due to incomplete oxidation of their sample. The difference between the *C. vinosum* spectrum observed in the present study and that of Stephens et al. (1978) can probably be attributed to an undetected baseline shift in one of the spectra. Confidence in the qualitative correctness of the *C. vinosum* spectrum in the present study is obtained from the fact that a large number of different HiPIPs display similarly shaped oxidized spectra (Figure 28).

The oxidized visible absorption spectrum of HiPIP is rather featureless with broad maxima at approximately 320, 400, 450 nm (Bartsch 1978; see Figure 3). The oxidized *C. vinosum* HiPIP spectrum exhibits negative maxima at ~ 346 nm and three positive maxima at 425, 488 and 580 nm. HiPIP spectra in groups B-D all appear to possess a negative maximum in the 320-350 nm region (*R. gelatinosa* being an exception) and in general display many spectral features in common with the spectra in group A. The apparent greater similarity of the oxidized visible spectra (versus the reduced state) among the different HiPIPs may result from differences in the cluster electronic or geometrical structure between the two oxidation states and/or the fewer polypeptide interactions that can occur as a result of the proposed cluster contraction that occurs upon oxidation (cf. Carter 1977b, see also pages 16 and 20).
Figure 28. Oxidized visible HiPIP CD spectra at pH 7.

Legend for spectra as in Figure 27, except for group B, where
Paracoccus sp. (---), *F. vacuolata* iso-1 (---), and *F. vacuolata*
iso-2 (-- -- --).
Near UV Reduced CD Spectra

In addition to the cluster absorbance (maximum ~ 280-300 nm; cf. Aizman and Case 1982), the protein has chromophores (aromatic amino acids) which contribute to the absorbance in the near UV wavelength region (250-320 nm; Figure 30). Thus, the CD in this region is quite complicated. The fact that the spectra differ in sign as well as magnitude suggests that extensive cancellation effects may be occurring.

In C. vinosum HiPIP, x-ray crystallographic studies have shown that all but one (TRP 60) of the aromatic residues (TYR 19, PHE 48, PHE 66, TRP 76, and TRP 80) within the protein are packed in close proximity to atoms of the cluster. Thus, it is probable that many of the aromatic residues and cluster transitions are strongly coupled to one another. The vibronic fine structure (cf. Strickland 1974) associated with aromatic side chain CD are apparent in the HiPIP near UV spectra (Figure 29). For HiPIPs with similar aromatic residue composition and sequence positions, qualitatively similar spectra are observed (cf. Group A Figure 29 and Table 1). From an examination of the various HiPIP sequences, the presence of a negative shoulder at ~ 290 nm appears to be associated with the presence of TRP 80, based on the present sequence alignment. HiPIPs which do not possess this spectral feature (Rp. globiformis, E. vacuolata iso-1, Rs. tenue 3761 and 2761 and the E. halophila iso-HiPIPs) do not have TRP 80 conserved (Table 1; Meyer 1984, personal communication). Iso-1-HiPIP from E. vacuolata differs in TRP content and sequence from E. vacuolata iso-2-HiPIP and group A HiPIPs at this site. It is interesting that the E. halophila iso-HiPIPs display a positive shoulder at ~ 290 nm. The
single additional TRP residue in these HiPIPs, not found in the others, is TRP 49. Consistent with the possible involvement of this residue in this apparently enantiomeric spectral features is that TRP 49 approaches the cluster from the opposite side of the effective mirror plane as compared to TRP 80 (Carter 1977b; see page 20). One might also speculate that the apparent enantiomeric relationship of the reduced visible spectral features between the *E. halophila* iso-HiPIPs and those in group A (see above) might also be due in part to electronic interactions of TRP 49 with the cluster. Deconvolution of the spectra would aide in the above empirical assignments.
Figure 29. Reduced near UV HiPIP CD spectra at pH 7.

Legend to spectra as in Figure 27.
**PH Perturbation of HiPIP Visible CD**

The suggested importance of interactions of amino acid side chain residues at position 49 and 80 with the cluster receives some support from the observation that *T. roseopersicina* HiPIP, which has a histidine at position 49 (Table 1), demonstrates a strong pH dependence on its oxidation reduction potential (see Table 7). *C. vinosum* HiPIP shows a much smaller effect (Table 7; Mizrahi et al. 1980). Since *T. roseopersicina* has another histidine (HIS 61) besides HIS 49, not found in common with *C. vinosum*, one cannot assign the pH effects observed with *T. roseopersicina* to HIS 49. However, based on the *C. vinosum* HiPIP structure, HIS 61 is much further removed from the cluster than HIS 49 (which is located within 5 A of the $S_4^*$ atom of the cluster; see Figure 2) and thus would be expected to exert less, if any, effect on redox potential. The effect of pH on redox potential is consistent with an electrostatic interaction of protonated form of the histidine residue with the negatively charged cluster, as has been previously suggested for the sole HIS 42 in *C. vinosum* and *Rg. gelatinosa* HiPIPs (Nettesheim et al. 1983). A small pH difference CD spectrum in the reduced form is observed for *T. roseopersicina* HiPIP which is different from those observed for *Rg. gelatinosa* and *C. vinosum* HiPIPs (Figure 31). The reduced oxidation state was studied, since, if an electrostatic interaction is involved, it would be expected to demonstrate a larger effect than the oxidized state because of its larger negative charge. Support that the observed difference spectra are due to histidine ionizations is obtained from the fact that reduced *Rs. tenue* 3761 HiPIP, which contains no histidines, produces no pH
difference spectrum. Feinberg and co-workers have suggested that that HIS 42 deprotonation in _C. vinosum_ and _R. gelatinosa_ HiPIPs is linked to environmental changes in TRP 76 and/or 80 (Nettesheim et al. 1980; see page 14). If TRP 80 is involved, then the presence of enantionmeric features in the difference CD spectra (Figure 30) would again be consistent with the opposite sides of the mirror plane being occupied by TRP 80 and HIS 49. These speculations require further investigation.
Figure 30. Reduced visible HiPIP pH 8.7 minus pH 5.1 difference CD spectra.

C. vinosum (——), R. gelatinosa (· · · · · · ·), T. roseopersicina (——)
Near UV Oxidized CD Spectra

As in the reduced state, aromatic vibronic structure is also apparent in the oxidized near UV CD spectra (Figure 31). The correlation between the negative shoulder at ~ 290 nm and TRP 80 (see above) is maintained in the oxidized state. As suggested by Flatmark and Dus (1969), the cluster probably accounts for a major portion of the differences observed between the near UV spectra for the two oxidation states. The general similarity of the oxidized minus reduced difference CD spectra for HiPIPs which demonstrate drastically different absolute CD spectra, supports this proposal (Figure 32). The differences observed between the spectra in Figure 32 are probably due to differences in the aromatic residue contributions.
Figure 31. Oxidized near UV HiPIP CD spectra at pH 7.
Legends for spectra as given in Figure 28.
Figure 32. Oxidized minus reduced HiPIP near UV difference CD.

*C. vinosum* (---), *R. gelatinosa* (•••••), *R. globiformis* (—•—), *E. halophila* iso-1 (— —)
Far UV Reduced CD Spectra

The far UV spectral region (210-250 nm) is characterized by contributions due to not only various amino acid side chains, and presumably the [4Fe-4S] cluster, but also the peptide bond (cf. Brahams and Brahams 1980). In instances where the protein has weak amide bands, aromatic side chains are suggested to make important contributions to this wavelength region (Woody 1978, Brahams and Brahams 1980). Based on the x-ray crystallographic studies of *C. vinosum* HiPIP, such a situation would be expected to apply to HiPIP. The negative band at ~230 nm in the *C. vinosum* spectrum (Figure 33) is similar in position to bands previously attributed to aromatic side chains (tyrosine and tryptophan; Woody 1978). Again, the group A HiPIPs display, as expected, very similar spectra in this region. It was previously suggested that the 230 nm band was associated with the cysteinyl coordination of the cluster (Flatmark and Dus 1969), however the finding that several HiPIPs do not possess this band (Figure 33) argues against this proposal and in favor of the involvement of aromatic residues. Significantly, the *E. halophila* iso-HiPIPs again display an approximate enantiomeric relationship to the group A spectra with regard to the ~230 nm band. This may result in part again from TRP 49 with negative maxima in other HiPIPs being attributed in part to TRP 80. The absence of negative maxima in the *Rs. tenue*, *Rp. globiformis*, and *E. vacuolata* iso-1 HiPIPs supports this speculation. All the spectra are consistent with little or no alpha-helix content.
Figure 33. Reduced far UV HiPIP CD spectra at pH 7.
Group A: C. vinosum (---), C. gracile (---),
T. roseopersicina (---); Group B: Paracoccus sp. (---), E. vacuolata
iso-1 (--.), E. vacuolata iso-2 (---);
Group C: Rp. gelatinosa (---), Rs. tenue 3761 (---); Group D: T.
pfennigii (---), Rp. globiformis (---),
E. halophila iso-1 (--.), E. halophila iso-2 (---)
Far UV Oxidized CD Spectra

The oxidized state far UV spectra demonstrate some differences from the reduced state for all HiPIPs. For *C. vinosum* HiPIP, the maximum at ~232 nm shifts to ~228 nm increasing slightly in intensity. There is also some small changes at lower wavelengths. These changes are probably attributed to cluster oxidation state differences and/or aromatic residue environment effects (cf. Nettesheim et al. 1980). Crystallographic studies have indicated only small conformational changes between the oxidized and reduced states of *C. vinosum* HiPIP (Carter et al. 1974a).
Figure 34. Oxidized far UV HiPIP CD spectra at pH 7.

Conclusions

No strict, simple correlation exists between reduced visible CD features and the utilization of the 3+,2+ [4Fe-4S] oxidation levels nor HiPIP oxidation-reduction potentials, as was suggested by Carter (1977b). The importance of amino acid side chains in close contact to the cluster in modifying spectral and oxidation-reduction properties are suggested. Specifically, the negative shoulder at ~290 nm and the negative maximum at ~230 nm are apparently associated with the presence of TRP 80. In *E. halophila* iso-HiPIS, which do not possess a TRP 80, the positive bands at ~290 nm and ~230 nm appear to be associated with TRP 49 which is located on the other side of the effective mirror plane of the cluster from TRP 80. The possible importance of position 49 is supported by *T. roseopersicina* HiPIP which has HIS 49 and the influence of pH on the redox potential and visible CD properties of this protein.
SUMMARY

Several factors are expected to affect the rate of biological electron transfer. In bimolecular reactions, electrostatics, nonpolar interactions, steric constraints, solvent reorganization, protein dynamics and conformation would all be expected to have a role in productive complex formation. The electron transfer step itself would be expected to be influenced by the distance between the redox active sites (i.e. the extent of orbital overlap), the characteristics of the intervening media between the redox centers (including the symmetry of the orbitals participating in the electron transfer), redox potential, and any required structural reorganizational processes. Laser flash photolysis and stopped-flow techniques have been utilized to quantitate the influence of a number of these factors on free and protein-bound flavosemiquinone oxidation by a variety of oxidants, although primarily a class of [4Fe-4S] ferredoxins referred to as HiPIPs.

The bimolecular oxidation of free flavosemiquinone was found to be influenced by electrostatics, sterics, redox potential, and possibly electronic effects. Quantitation of all four factors was possible.

The effect of pH on the reaction of free flavosemiquinone analogs generated by laser-flash photolysis with oxidized Chromatium vinosum high potential iron-sulfur protein, other iron-containing redox proteins and with non-biological one-electron oxidants was investigated. The results demonstrate that the second order rate constant for the oxidation of lumiflavin flavosemiquinone increases
dramatically with increasing pH for the redox proteins (C. vinosum HiPIP ~ 63 fold, C. pasteurianum Rd ~ 43 fold, and tuna cytochrome c ~37 fold over a pH range from 5 to 10) and some of the other oxidants (Co(phen)$_3^{3+}$ ~ 72 fold and FeEDTA ~ 48 fold over a pH range 5.0-7.8). The pH-rate constant profiles for the redox proteins closely follow the ionization of the proton at the N-5 position of the neutral lumiflavin flavosemiquinone (pK 8.4), suggesting a higher intrinsic reactivity for the anionic lumiflavin flavosemiquinone. This increased reactivity could result from changes in electron spin density distribution between the two protonic forms of the semiquinone (i.e. electronic effects). Similar pH dependencies are observed for a number of flavin structural analogs, yielding estimates of the N-5 pK values for these analogs.

The second order rate constants for oxidation of various flavosemiquinone analogs at different pH values (where either the neutral or anionic flavosemiquinone predominates) by either redox proteins or non-biological oxidants correlate with the difference in redox potential of the reactants according to the Marcus exponential equation. For the protein–flavin analog reactions, deviations from the theoretical Marcus curve are interpreted in terms of the effects of the different exocyclic substitutions on intrinsic anion semiquinone reactivity. Possible steric or solvation effects from the ribityl chain of riboflavin results in ~30% decrease in reactivity as compared to lumiflavin. Chlorine-substituted (at the 7 and 8 positions) flavosemiquinone analogs indicate changes in reactivity which are probably related to differences in electron spin density distribution. The data are consistent with the involvement of both the
N-5-dimethylbenzene ring portion and the C-4a position of the flavin in flavosemiquinone oxidation by one-electron oxidants. The increased intrinsic reactivity of the anionic flavosemiquinone could be utilized as a component of the reaction mechanisms of flavoproteins containing covalently-bound flavin, which are known to produce the anionic flavosemiquinone at physiological pH values.

Electrostatic effects on FMN and C. pasteurianum flavodoxin semiquinone oxidation by HiPIP can be quantitated using a parallel plate bimolecular electrostatic equation developed by Watkins (Watkins 1984). The magnitude of the electrostatic effects are larger for flavodoxin semiquinone oxidation than for FMN semiquinone oxidation which is consistent with the larger electrostatic charge for flavodoxin (e.g. with C. vinosum HiPIP the rate constant increases ~ 9 fold for the flavodoxin reaction and ~ 3 fold for the FMN reaction over an ionic strength over an ionic strength range 0.014-0.49 M). Calculated charges at the electrostatic interaction site on HiPIP for both the flavodoxin and FMN reactions vary with expectation, based on sequence substitutions among the HiPIPs at a localized region of the protein where the cluster closely approaches the protein surface. This site was previously suggested to be involved in the iron hexacyanide redox reactions with HiPIP (Mizrahi et al. 1980).

The $k_0$ values obtained from the electrostatic analysis for FMN semiquinone and flavodoxin semiquinone oxidation indicate a dependence on the redox potential difference between the reactants ($\Delta E$). The $\Delta E$ effect is larger in magnitude for the flavodoxin reaction than for the FMN reaction (~ 300 fold versus ~ 3 fold over a 0.21 V $\Delta E$ range). The
dependence of $k_D$ on $AE$ for the FMN reaction can be fit to the Marcus and Hopfield equations. A comparison with lumiflavin semiquinone oxidation results at pH 7 suggests that the apparent lower intrinsic reactivity of FMN semiquinone ($\sim 48\%$) results in part from its higher $pK$ (8.6) and from steric or solvation effects of the ribityl group (and perhaps the phosphate). For the flavodoxin results, the data do not appear to give adequate fits to either the Marcus and Hopfield equations. This suggests that factors other than redox potential and electrostatics, such as sterics, which were not taken into consideration in the present analysis, could be having an important influence on reactivity. The results suggest that electrostatics, redox potential, and perhaps sterics could have an important role in determining the biological specificity of protein/protein redox reactions.

Absorption difference spectroscopy, difference CD, and gel filtration column chromatography indicate that oxidized *C. pasteurianum* rubredoxin ($\text{Rd}_{\text{ox}}$) and oxidized spinach ferredoxin:NADP+-reductase ($\text{FNR}_{\text{ox}}$) form a 1:1 complex at low ionic strength (10 mM), which is completely dissociated at higher ionic strength (310 mM). Apparent second order rate constants for the oxidation of lumiflavin semiquinone by $\text{Rd}_{\text{ox}}$ in its free and complexed state are the same ($\sim 1.3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$).

Reduction of $\text{Rd}_{\text{ox}}$ (both free and complexed) by free FNR semiquinone and intracomplex electron transfer were investigated using 5-deazariboflavin semiquinone as the reductant. At $I = 10$ mM, for increasing amounts of a 1:1 mixture of $\text{Rd}_{\text{ox}}/\text{FNR}_{\text{ox}}$ mixtures, a first
order rate constant of $2.0 \times 10^3 \text{ s}^{-1}$ was obtained, which corresponds to the processes involved in intracomplex electron transfer from FNR semiquinone to $\text{Rd}_{\text{ox}}^-$. This relatively large rate constant for the intracomplex reaction could result from the low reorganizational energy for the reduction of $\text{Rd}_{\text{ox}}^-$, the relative exposure of the Fe center and FAD (i.e. the extent of orbital overlap or distance effects) or the large thermodynamic driving force for the reaction. Additional examples of intracomplex electron transfer involving FNR semiquinone are required to determine which factors are most important. At high $\text{FNR}_{\text{ox}}^+/\text{Rd}_{\text{ox}}$ ratios, a second order reaction between free FNR semiquinone and complexed $\text{Rd}_{\text{ox}}$ was also observed to occur ($k = 5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). At $I = 310$ mM, these reactions are not observed and the reaction of FNR semiquinone with free $\text{Rd}_{\text{ox}}$ is second order ($k = 4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$).

Since conformation of a reactant could influence electron transfer reactions, a survey of the CD properties of HiPIP was undertaken. Drastic differences in the CD of HiPIPs are observed throughout the far and near UV and visible spectral regions. No strict simple correlation between HiPIP redox behavior and the reduced visible CD is observed, as was suggested by Carter (1977b). Substitutions of specific amino acids could account for differences in certain spectral features for the various HiPIPs. Specifically, the negative shoulder at ~290 nm and the negative maximum at ~230 nm apparently are associated with the presence of TRP 80 which closely interacts with an inorganic sulfur of the cluster. In the *E. halophila* iso-HiPIPs, which do not possess a TRP 80, the positive band at ~290 nm and ~230 nm appears to be associated with TRP 49. The tryptophan residue (based
on the *C. vinosum* HiPIP structure) is located near a cluster inorganic sulfur that is on the opposite side of the effective mirror plane of the cluster from the TRP 80 interaction. The importance position 49 in interactions with the cluster is supported by observations on *T. roseopersicina* HiPIP, which has a HIS 49. The influence of pH on the redox potential and visible CD properties of this protein as well as of ionic strength on kinetic studies suggests that HIS 49 is electrostatically and electronically coupled to the cluster.
## APPENDIX A. STRUCTURES AND $E_{M,7}^{\text{nV}}$ VALUES FOR FLAVIN ANALOGS

### Ring Substituents

<table>
<thead>
<tr>
<th>Flavin Analog (Abbreviation)</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$E_{M,7}^{\text{nV}}$ ($\text{mV}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-methyl-isoalloxazine (10-MI)</td>
<td>$-\text{H}$</td>
<td>$-\text{H}$</td>
<td>$-\text{CH}_3$</td>
<td>$-167^a$</td>
</tr>
<tr>
<td>Lumi-flavin (Lf)</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{CH}_3$</td>
<td>$-207^a$</td>
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<tr>
<td>Riboflavin (Rf)</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{ribityl}$</td>
<td>$-208^b$</td>
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<tr>
<td>7-chlororiboflavin (7-CIRf)</td>
<td>$-\text{Cl}$</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{ribityl}$</td>
<td>$-128^b$</td>
</tr>
<tr>
<td>8-chlororiboflavin (8-CIRf)</td>
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<td>$-\text{Cl}$</td>
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<td>$-144^b$</td>
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<tr>
<td>7,8-dichlororiboflavin (7,8-diCIRf)</td>
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<td>$-\text{Cl}$</td>
<td>$-\text{ribityl}$</td>
<td>$-95^a$</td>
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<tr>
<td>$8\alpha(-S$-mercaptopropionic acid) riboflavin (thioether) (8-MP-T)</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{CH}_2-S-(\text{CH}_2)_2\text{COO}^-$</td>
<td>$-\text{ribityl}$</td>
<td>$-174^c$</td>
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<tr>
<td>$8\alpha(-S$-mercaptopropionic acid) riboflavin (sulphone) (8-MP-S)</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{CH}_2-S-(\text{CH}_2)_2\text{COO}^-$</td>
<td>$-\text{ribityl}$</td>
<td>$-$</td>
</tr>
<tr>
<td>5-deazariboflavin (5-dRf)$^2$</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{CH}_3$</td>
<td>$-\text{ribityl}$</td>
<td>$-650^d$</td>
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</tbody>
</table>

1. Positions of $R$ groups as given in following:

2. The flavin ring system for this analog has N-5 replaced with CH.

3. The redox potentials are for the oxidized/fully reduced couple in all cases except for 5-dRf for which the oxidized/semiquinone redox potential is given.

---

$a$ Perzer and Radda 1971.

$b$ Walsh et al. 1978.

$c$ For $8\alpha-N$-acetylated cysteinyl-Rf; Falk et al. 1976.

$d$ Blanekhorn 1976.
APPENDIX B. HIPIP ABSORPTION SPECTRAL PROPERTIES

<table>
<thead>
<tr>
<th>HiPIP Source</th>
<th>Purity Index oxidized state</th>
<th>$\epsilon_{\text{red vis max}}$ $\text{M}^{-1}\text{cm}^{-1}$</th>
<th>$\Delta\epsilon_{500\text{ nm}}$ $\text{M}^{-1}\text{cm}^{-1}$</th>
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<tbody>
<tr>
<td>C. vinosum (O)</td>
<td>2.2$^a$</td>
<td>16.1x10$^3$</td>
<td>9.3x10$^3$</td>
</tr>
<tr>
<td>T. roseopersicina (L)</td>
<td>2.2$^e$</td>
<td>14.7x10$^3$ d</td>
<td>8.2x10$^3$</td>
</tr>
<tr>
<td>C. gracile (K)</td>
<td>2.2$^e$</td>
<td>---</td>
<td>9.7x10$^3$</td>
</tr>
<tr>
<td>T. pfennigii (P)</td>
<td>2.3$^b$</td>
<td>15.3x10$^3$ b</td>
<td>8.3x10$^3$</td>
</tr>
<tr>
<td>Rp. galatinosa (O)</td>
<td>1.8$^a$</td>
<td>15.3x10$^3$ a</td>
<td>8.8x10$^3$</td>
</tr>
<tr>
<td>Paracoccus sp. (J)</td>
<td>1.7$^c$</td>
<td>14.8x10$^3$ c</td>
<td>7.0x10$^3$</td>
</tr>
<tr>
<td>E. vacuolata iso-2-HP (T)</td>
<td>2.2$^e$</td>
<td>---</td>
<td>8.8x10$^3$</td>
</tr>
<tr>
<td>E. vacuolata iso-1-HP (S)</td>
<td>1.9$^e$</td>
<td>---</td>
<td>8.8x10$^3$</td>
</tr>
<tr>
<td>E. halophila iso-2-HP (V)</td>
<td>2.2$^e$</td>
<td>---</td>
<td>8.6x10$^3$</td>
</tr>
<tr>
<td>E. halophila iso-1-HP (U)</td>
<td>2.1$^e$</td>
<td>---</td>
<td>8.6x10$^3$</td>
</tr>
<tr>
<td>Rs. tenue 2761 (R)</td>
<td>1.1$^e$</td>
<td>---</td>
<td>8.4x10$^3$</td>
</tr>
<tr>
<td>Rs. tenue 3761 (N)</td>
<td>1.1$^e$</td>
<td>16.9x10$^3$ c</td>
<td>8.4x10$^3$</td>
</tr>
<tr>
<td>Rp. globiformis (M)</td>
<td>1.8$^f$</td>
<td>---</td>
<td>6.6x10$^3$</td>
</tr>
</tbody>
</table>

$^a$Dus et al. 1967.
$^c$Bartsch 1978.
$^d$Zorin and Gogotov 1984.
$^e$This work.
$^f$Since we were unable to fully oxidize the protein; the purity index given is for the reduced oxidation state.
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