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THE ROLE OF HISTIDINE-42 IN THE OXIDATION-REDUCTION MECHANISM OF *CHROMATIUM VINOSUM* HIGH POTENTIAL IRON-SULFUR PROTEIN

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Summary

The second order rate constants for the oxidation of high potential iron-sulfur protein (Hipip) of *Chromatium vinosum* by ferricyanide were determined as a function of ionic strength and pH. From the ionic strength results, calculations were done to correct the rate constant at each pH for the electrostatic interactions between Hipip and ferricyanide. The electrostatic corrections are necessary since the charge of the protein changes as a function of pH and can mask the ionization of mechanistically important amino acid residues. An apparent $pK_a \simeq 7$ was obtained from electrostatically corrected rate-pH profile, indicating the possible participation of histidine. Perturbation difference spectroscopic studies of Hipip as a function of pH also gave apparent pK_a values of 6.9 and 6.7 for the reduced and oxidized protein, respectively. That it was indeed His 42 (the only His in the polypeptide) that was responsible for the kinetic and spectroscopic pK_a values was demonstrated by modification of His 42 of Hipip by the histidine selective reagent diethylpyrocarbonate. No modification of Tyr 19 could be detected. It is concluded that either deprotonation or modification of His 42 results in the destabilization of the reduced cluster and thus a faster rate of oxidation. This work provides the first experimental evidence of the 'squeeze effect' mechanism (Carter, C.W., Jr., Kraut, J., Freer, S.T. and Alden, R.A. (1974) J. Biol. Chem. 249, 6339—6346) in which the polypeptide directly modulates the stability of the iron-sulfur cluster.

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Abbreviation: Hipip, high potential iron-sulfur protein.

Introduction

Iron-sulfur proteins play an important role in the electron transfer processes of terminal respiration, photosynthesis, and nitrogen fixation. A particularly interesting class of iron-sulfur proteins are those which have the $Fe_4S_4^*$ (S^* = inorganic sulfide) cluster as the prosthetic group which transfers only $\pm 1e^-$ electron. The high potential iron-sulfur proteins (Hipip), which are now classified as high potential ferredoxins (Fd), pose an interesting problem in protein redox chemistry since the $E^{0'}_{Hipip} \simeq +350$ mV while the more typical ferredoxins have $E^{o}_{Fd} \simeq -430$ mV. A three state hypothesis has been proposed to explain these differences in redox potential [1], and too, there is evidence that the cluster can, under certain conditions, undergo two separate one electron transfers [2]. More generally the large differences in the redox potentials (and thus function) between Hipip and ferredoxin have been attributed to subtle but nonetheless significant protein structural differences within the interior of the protein. More recently Adman et al. [3] have found in a comparison of X-ray crystallographic structures, that there are more internal -N-H · · · S-bonds (18 vs. 6) in the *Peptococcus aerogenes* ferredoxin structure than in Hipip. Along the same lines it has been postulated by Carter et al. [4] that possibly the protein itself modulates the geometry, and thus, the properties of the Fe_4S_4 cluster. This is in clear contradiction to the more generally accepted position that the prosthetic group determines the tertiary structure, though these effects are interdependent on each other.

Although *Chromatium vinosum* Hipip has been well characterized with respect to both its structure [5], amino acid sequence [6] and other biophysical properties [7,8], its mechanism of electron transfer is still poorly understood. More critically there has been, to date, no evidence to redox link any particular amino acid residue of the primary structure or location on the protein surface to the electron transfer process.

In this work we report detailed spectroscopic and kinetic studies of native and modified high potential iron-sulfur proteins of both *C. vinosum* and *Rhodopseudomonas gelatinosa*. The results of absorbance difference spectroscopy of native and diethylpyrocarbonate-modified Hipip are correlated to redox state and kinetic behavior of the proteins. Lastly, in regard to the kinetic studies of the ferricyanide oxidation of Hipip, a novel use of electrostatically corrected second order rate constants is employed to partition electrostatic effects from the ionization of specific and mechanistically important amino acid residues.

Materials and Methods

Potassium ferricyanide and sodium dithionite, were obtained from Fisher; diethylpyrocarbonate, 4-chloro-3,5-dinitrobenzoic acid, and 2-mercaptoethanol were obtained from Aldrich, and Tris base from Sigma. All other chemicals used were of reagent grade and only double distilled water was used. All pH measurements were made with a Corning Model 12 pH meter with Corning 476050 combination electrode. All spectrophotometric measurements, excluding stopped flow spectrophotometry, were made with a Varian Cary 17 spectrophotometer.

Chromatium vinosum and *Rhodopseudomonas gelatinosa* Hipips were isolated and purified as described by Bartsch [9,10], Tedro et al. [11], and DeKlerk and Kamen [12]. For the kinetic experiments extinction coefficients determined by Dus et al. [7] were used: 41.3 mM and 16.1 mM for reduced Hipip at 283 nm and 388 nm, respectively; 39.3 mM and 21.8 mM for oxidized Hipip at 283 and 325 nm, respectively. The extinction coefficient reported by Kortüm [13] of 742.4 M^{-1} at 436 nm was used for ferricyanide.

Stopped flow spectrophotometric kinetics. Kinetic studies, done at different pH values and ionic strengths, were carried out with a Durrum stopped flow spectrophotometer using a pneumatic air actuated pushing device. Absorption measurements were made with a linear optical configuration utilizing a tungsten lamp source and 2 cm light path. Phototube voltages were stored in a Northern Scientific NS-560 time averaging computer modified to collect data at two scanning speeds in each of two 1024 memory cell storage units. The data were then hard-copy plotted with a Houston Instrument Omnigraphic 2000 X-Y Recorder. Data were treated by a regression analysis of the linear form of the first order rate law. Second order rates were determined under pseudo-first order conditions using a series of increasing ferricyanide concentrations.

All buffers were deaerated 30 min with nitrogen that had passed through an oxygen scrubbing tower. For buffers with pH values of 10.15 and 11.3 a drying tube containing sodium hydroxide was attached atop a miriad bottle. Stock solutions of reduced Hipip were prepared by the addition of a slight excess of dithionite, and passage over a small Sephadex G-25 column preequilibrated at the appropriate pH and ionic strength. A sample of buffer was taken at the time of application to the Sephadex column and the pH measured. After passage over the Sephadex column the protein was collected in a ground-glass stoppered vial and diluted to the appropriate concentration with buffer. In all cases at each pH and ionic strength, the rate of the reaction was measured at three different excesses (10-fold and greater) of ferricyanide to demonstrate second order kinetics and no binding effects.

Amino acid residue modifications. Histidine modification was accomplished with the histidine selective reagent diethylpyrocarbonate in phosphate buffer (50 mM), pH 6.0. Diethylpyrocarbonate was added neat in greater than 100 × molar excess.

Electrostatic corrections. The kinetic-ionic strength data were analyzed by a non-linear least squares regression analysis of Eqn. 1 derived by Wherland and Gray [14] from the Marcus theory of outer sphere electron transfer reactions and Debye potential theory:

$$\ln k_I = \ln k_\infty - 3.576 \times \left[\frac{e^{-\kappa R_A}}{1+\kappa R_B} + \frac{e^{-\kappa R_B}}{1+\kappa R_A}\right]\left[\frac{Z_A \cdot Z_B}{R_A + R_B}\right] \quad (1)$$

where k_I is the observed rate constant at ionic strength I, Z_A and Z_B are the net charges of the reactants of A and B, respectively, R_A and R_B are the radii of A and B, respectively, $\kappa = 0.329\ I^{1/2}$ Å^{-1}, and k_∞ is the rate constant at infinite ionic strength (i.e., the rate that is independent of electrostatic interactions since the charges of reactants are fully shielded from each other by the ions of the electrolyte solution). The rate constant at infinite ionic strength is the

electrostatically corrected rate constant, k_∞. From the curve fit both the apparent net charge of the protein and k_∞ are obtained. As an additional verification of the validity of Eqn. 1, the predicted redox protein charge from Eqn. 1 was compared to the net charge of the protein as determined from the sequence at a particular pH.

Perturbation difference spectra of Hipip at different pH values and under conditions of diethylpyrocarbonate modification were obtained with a Cary 17 spectrophotometer at 0.1 A full scale. Both K_2 and ΔA_0 were obtained from the best least squares fit to Eqn. 2 [15]:

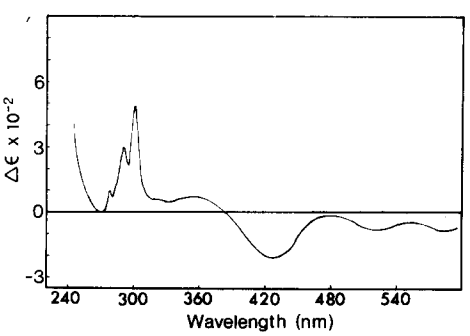
$$\frac{1}{\Delta A} = \frac{[H^+]}{K_2 \, \Delta A_0} + \frac{1}{\Delta A_0} \tag{2}$$

where K_2 is the proton dissociation constant and ΔA_0 is the absorbance which would be measured upon complete proton dissociation. Titrations were carried out with 0.05 M KOH in 100 mM KCl, following the principle peak ($\simeq$300 nm) of the aromatic perturbation, which increased upon increasing pH.

Results

In Fig. 1 is shown a pH perturbation difference spectrum of Hipip at pH 7.3 (sample) vs. pH 6.0 (reference). There are significant changes in both the aromatic region and also visible region of the spectrum where the $Fe_4S_4^*$ cluster absorbs. As the pH was made more basic (i.e., larger ΔpH) the aromatic perturbations also increased and were followed for titration purposes. The entire difference perturbation-pH study for both oxidized and reduced protein are shown in Fig. 2. The pK_a calculated from Eqn. 2 are 6.9 and 6.7 for reduced and oxidized Hipip, respectively.

Since a $pK_a \simeq 7$ was obtained from the ΔA vs. pH studies, histidine specific modification by diethylpyrocarbonate [16] of the single histidine-42 residue (possibly also a certain number of surface lysines) of Hipip (both *C. vinosum*



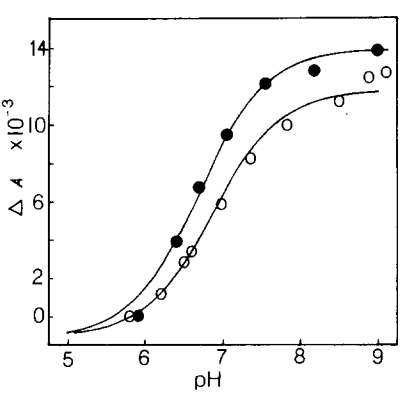


Fig. 1. pH perturbation difference spectrum of reduced Hipip pH 7.3 vs. pH 6.0; Hipip, $1.3 \cdot 10^{-5}$ M in 100 mM KCl.

Fig. 2. Plot of ΔA vs. pH for both reduced (•) and oxidized (○) Hipip with pK_a = 6.9 and 6.7, respectively.

and *Rps. gelatinosa*) was carried out to test whether or not the sole His-42 residue was responsible for the observed spectroscopic changes. In Fig. 3 is shown the difference spectrum of $HiPIP_{red}$ after its in situ modification with diethylpyrocarbonate. The maximum ΔA obtained from the ΔA vs. pH is about the same magnitude of the ΔA obtained upon diethylpyrocarbonate modification ($\Delta A \simeq 1.3 \cdot 10^{-2}$). Unfortunately it is not possible to use protein chemical methods to demonstrate unequivocally that it is only the His-42 being modified, since the mono-diethylpyrocarbonate-modified histidine is a relatively labile product [16,17]; however, it is known that of the various groups that can be modified by diethylpyrocarbonate, only the diethylpyrocarbonate-modified histidine or tyrosine product is reversed by treatment with hydroxylamine- and not diethylpyrocarbonate-modified lysines [17]. In this work the spectroscopic evidence indicated that the tyrosine residue was not modified under our experimental conditions. It was further established that the diethylpyrocarbonate modification was indeed the modification of histidine by reversing most of the diethylpyrocarbonate modification with hydroxylamine and observing the concomitant decrease of $\Delta\epsilon$ in the difference spectrum (i.e. with the exception of about 15% of the 236 nm band left, the spectrum returns to that of native Hipip). However, the remaining difference spectrum in the region of 320—560 nm indicated that some autooxidation of protein may have occurred because of possible destruction of His-42 [18] or the modification per se (see Fig. 3).

An additional set of experiments was done in which difference spectra were obtained for diethylpyrocarbonate $Hipip_{ox}$ vs. native $Hipip_{ox}$ (see Fig. 4). Again the important difference peaks are observed in the aromatic and to a lesser extent in the visible region; furthermore, this experiment with oxidized protein excludes the possibility that these differences are due to autooxidation. Upon addition of mercaptoethanol to reduce both reference and sample, the aromatic perturbations are shifted to the red while the visible perturbations increase in magnitude and are identical to the difference spectrum of native

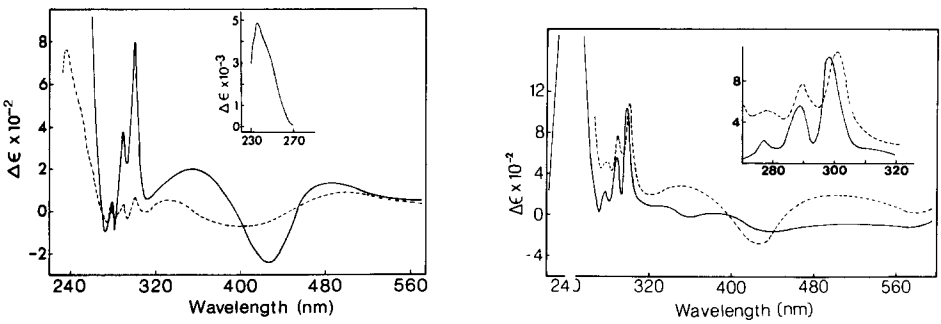
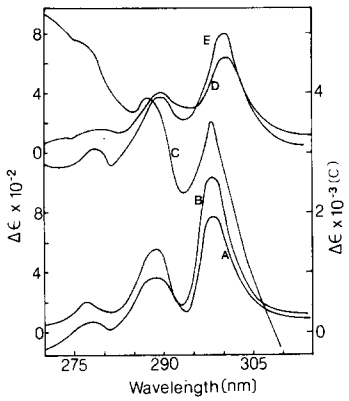


Fig. 3. Perturbation difference spectra of diethylpyrocarbonate-modified reduced Hipip vs. native reduced Hipip ($2 \cdot 10^{-5}$ M) before (solid line) and after reversal of diethylpyrocarbonate modification by NH_2OH, 20 mM (dashed line), pH 6.0. Hydroxylamine added to both reference and sample cuvettes.

Fig. 4. Perturbation difference spectrum of diethylpyrocarbonate-modified oxidized Hipip vs. native oxidized Hipip (solid line) followed by reduction of both reference and sample by 2-mercaptoethanol (dashed line). Insert shows more clearly the red shift of the spectrum after reduction.



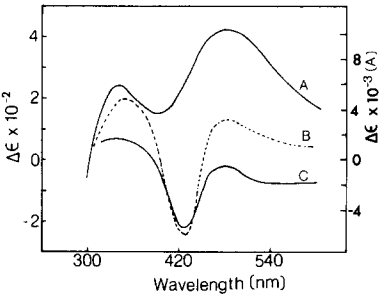


Fig. 5. Comparison of perturbation difference spectra and oxidized-reduced difference of Hipip in aromatic region. (A) Oxidized Hipip; pH 8.2 vs. 5.9. (B) Oxidized Hipip; diethylpyrocarbonate-modified vs. native. (C) Native reduced Hipip vs. native oxidized Hipip (pH 6.0). (D) Reduced Hipip, pH 7.8 vs. 5.8. (E) Reduced Hipip, diethylpyrocarbonate-modified vs. native.

Fig. 6. Comparison of perturbation difference spectra and oxidation-reduction difference spectrum in the visible ('cluster') region. (A) Oxidized-reduced difference spectra. (B) Reduced diethylpyrocarbonate-modified Hipip vs. native. (C) Reduced Hipip, pH 7.3 vs. 6.0.

TABLE I

SECOND ORDER RATES OF OXIDATION OF *CHROMATIUM* Hipip BY FERRICYANIDE

	Ionic strength	k ($M^{-1} \cdot s^{-1}$) $\times 10^{-3}$		Ionic strength	k ($M^{-1} \cdot s^{-1}$) $\times 10^{-3}$
Native Hipip			**Native Hipip**		
pH 5.0	0.0051	1.05	pH 9.35	0.0298	0.800
	0.0351	1.60		0.0598	1.35
	0.651	2.13		0.0898	2.06
	0.1051	2.80		0.1298	2.52
	0.2051	3.51		0.2298	3.87
pH 6.0	0.0056	0.422	pH 10.15	0.0151	0.477
	0.0560	1.42		0.0451	1.31
	0.1560	2.80		0.0751	1.89
	0.2560	3.42		0.1151	2.37
pH 7.0	0.0088	0.546		0.2151	3.35
	0.0338	1.23	pH 11.3	0.0166	0.391
	0.0588	1.68		0.0466	0.937
	0.1088	2.41		0.0766	1.43
	0.1588	3.05		0.1166	1.91
	0.2088	3.30		0.2166	3.28
pH 7.95	0.0136	0.506			
	0.0436	1.30			
	0.0736	1.85			
	0.1136	2.54			
	0.2136	3.66			
Diethylpyrocarbonate-modified Hipip					
pH 6.0	0.0056	0.422			
	0.0560	1.85			
	0.1560	3.36			
	0.2500	4.04			

TABLE II

ELECTROSTATICALLY CORRECTED SECOND ORDER RATES k_∞ AS A FUNCTION OF pH FOR NATIVE AND DIETHYLPYROCARBONATE-MODIFIED Hipip

	pH	k_∞ ($M^{-1} \cdot s^{-1}$) X 10^{-3}
Native Hipip	5.0	4.1
	6.0	4.7
	7.0	5.1
	8.0	6.2
	9.4	6.6
	10.2	6.0
	11.3	5.5
Diethylpyrocarbonate-modified Hipip	6.0	6.0

reduced vs. diethylpyrocarbonate modified reduced Hipip. The results for the pH and diethylpyrocarbonate modification perturbation difference spectra are compared with the oxidized-reduced difference spectrum in Figs. 5 and 6. The spectral shifts are systematic.

The Hipip of *Rps. gelatinosa* also has only one histidine 42, using *Chromatium* sequence numbering [19]. The perturbation difference spectra were the same as those observed for *C. vinosum* Hipip. Upon long reaction times both diethylpyrocarbonate-modified Hipips undergo apparent oxidation. When Hipip is permitted to react with the lysine modifying 4-chloro-3,5-dinitrobenzoic acid, no such oxidation was observed, thus demonstrating indirectly that it is the modification of the histidine 42 that generates the observed structural and functional changes.

In obtaining the functional counterpart to this study, the kinetics of ferricyanide oxidation of native Hipip and diethylpyrocarbonate-modified Hipip were determined (see Table I). The need for electrostatic corrections for redox protein reactions has been shown by Feinberg and coworkers [20—24]. The electrostatically corrected rates are summarized in Table II and plotted vs. pH in Fig. 7, providing apparent pK_a values of 7 and 10. To show the consistency of k_∞ with the net charge of the protein, the net charge of the protein as calculated by Eqn. 1 is compared to the net charge based on the primary

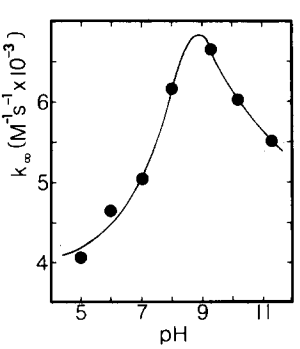


Fig. 7. Plot of electrostatically corrected second order rates of oxidation of Hipip by ferricyanide as a function of pH.

TABLE III

COMPARISON OF CALCULATED NET PROTEIN CHARGE WITH CHARGE BASED ON PRIMARY SEQUENCE AND CLUSTER AT DIFFERENT pH VALUES

pH	$Z_{Eq.1}$	$Z_{seq.}$
5.0	−2.0	−2.2
6.0	−3.4	−3.4
7.0	−3.6	−3.9
7.95	−4.5	−4.1
9.35	−5.2	−5.3
10.15	−4.7	−7.0
11.3	−5.2	−10.2

sequence [6] in Table III. The agreement is excellent except at higher pH values and confirms the fact that both pH and ionic strength modulate the observed second order rates [20—24].

More critically the electrostatically corrected rate constant for the diethylpyrocarbonate-modified Hipip was found to be $6.02 \cdot 10^3\ M^{-1} \cdot s^{-1}$, which compares well with the maximum observed rate of about $6.7 \cdot 10^3\ M^{-1} \cdot s^{-1}$.

Discussion

In Fig. 1 is shown the pH perturbation difference spectrum which is the result of a red shifted difference spectrum [25]. This kind of difference spectrum with peaks at approx. 300 nm, 290 nm and 280 nm is typical of the solvent perturbation difference spectra of model tryptophan compounds taken to more hydrophobic environments [26]. In this work the difference spectra are even further red shifted and indicate that indeed the Hipip tryptophans are in a relatively more hydrophobic environment in the native protein than the model compounds. This is expected in part since X-ray structure of Hipip shows that Trp 76 and 80 are well buried in the hydrophobic protein interior and are both near the $Fe_4S_4^*$ cluster [5]. Furthermore in studies of proteins with tryptophan residues on the protein surface [27] the spectral red shifts were also observed as the environment of the tryptophans was made more hydrophobic with the appropriate solvent such as dimethylsulfoxide or ethylene glycol. In the latter work, which depends on changes in bulk solvent, only external tryptophans are observed in the difference spectra via solvent perturbation and the perturbations occur at about the same wavelengths as those expected for free tryptophan [27]. In contrast, in this work, the solvent at least in regard to its bulk polarity is not changed, but rather the environment of the interior of the protein is altered through the deprotonation of what will be shown to be histidine-42. The perturbations at approx. 300 nm, 290 nm, and 280 nm are assigned to tryptophan and not tyrosine since tryptophan produces a triplet of peaks rather than a doublet for tyrosine [26,28] and His-42 is in close proximity to Trp 80 but not Tyr 19 [5]. It is also unlikely that the ionization of Tyr 19 occurs, since even at high ΔpH the strong ultraviolet absorption at 250 nm is not observed [29]. Thus pH perturbation differences in the aromatic region can be reasonably ascribed to microenvironmental changes of tryptophan residues. In examining the pH perturbation

difference spectroscopic titration with both oxidized and reduced Hipip (see Fig. 2), pK_a values of 6.9 and 6.7 were observed for reduced and oxidized protein, respectively. Both pK_a values are what one would reasonably expect for the histidine residue and the difference is clearly a function of redox state; in contrast the p*I* values of the two reduced Hipips are 3.68 (*C. vinosum*) and 9.50 (*Rps. gelatinosa*).

In order to demonstrate that the titration in Fig. 2 reflects the deprotonation of His-42, native oxidized and reduced Hipip were modified by diethylpyrocarbonate (see Figs. 3 and 4). Upon modification strong perturbations are again observed at approx. 300 nm, 290 nm and 280 nm and are of the same magnitude as the pH perturbation difference spectra in a given redox state. The appearance of the intense ($\epsilon = 4500$) 236 nm band is typical of diethylpyrocarbonate-modified histidine [16]; however, tryptophan itself also makes some contribution at 236 nm under conditions of perturbation [30] and this explains why the extinction coefficient at 236 is $\epsilon \simeq 4500$ rather than the expected $\epsilon \simeq 3200$ [16]. After correction for the tryptophan contribution at 236 nm, the calculated stoichiometry of diethylpyrocarbonate modification was 0.9 diethylpyrocarbonate per Hipip. Further evidence that it is indeed the His-42 that is responsible for the aromatic perturbations is that most of the 236 nm band and the aromatic perturbations are decreased dramatically upon the reversal of the diethylpyrocarbonate modification with hydroxylamine (see Fig. 2). Hydroxylamine only reverses singly modified histidine and tyrosine [16,17] and not lysines, etc. In addition to having a vastly different pK_a from that observed in the pH perturbation difference spectra, tyrosine-19 is an unlikely candidate for diethylpyrocarbonate modification since the observed spectroscopy does not correspond to that published for model studies of tyrosine [31]. What is remarkable is that the deprotonation of His-42, either by pH increase or diethylpyrocarbonate modification, results in significant changes of the tryptophan environment as reflected in the perturbation difference spectra.

In Fig. 5 a comparison is made between three classes of spectra: (1) diethylpyrocarbonate modification perturbation differences, both redox states, (2) pH perturbation differences, both redox states, and (3) reduced vs. oxidized Hipip. The common spectral difference in all of these perturbations is the triplet of peaks at 300 nm, 290 nm, 280 nm. What is notable and important in Fig. 5 is that: (1) in going from the oxidized to the reduced state, the triplet is observed and (2) upon deprotonation or diethylpyrocarbonate modification the triplet is observed and red shifted even further when taken from the oxidized to reduced state. This is also seen in Fig. 4. The red shifts observed indicate that the tryptophan(s) are in a more hydrophobic environment in the reduced state.

In further making the assignment of the triplet of peaks in the difference spectra as shown in Fig. 5, an important comparison can be made between their oxidized-reduced difference spectra of various Hipips. In those Hipips (*Chromatium vinosum*, *Rps. gelatinosa* and *Paracoccus* sp.) where the tryptophans 76 and 80 are fully conserved, a strong perturbation at about 300 nm is observed [9—11]. Only in the case of *Rhodospirillum tenue* Hipip, in which both Trp 76 and 80 have been replaced by tyrosine, is it missing [10,19].

In Fig. 6, the oxidized vs. reduced difference spectrum is compared to both the pH and diethylpyrocarbonate modification perturbations in the visible region of the spectrum where the $Fe_4S_4^*$ absorbs. The difference spectroscopy indicates that either deprotonation or diethylpyrocarbonate modification of His-42 causes the cluster to resemble spectroscopically the oxidized state, in spite of the fact the proteins are in the reduced state for both experiments. The same perturbations were observed when the modification was carried out in the presence of dithiothreitol, which insured that the Hipip was in the reduced state.

Further support that deprotonation or diethylpyrocarbonate modification of His-42 destabilizes the reduced state of Hipip (i.e., favors oxidized $Fe_4S_4^*$) comes from the electrostatically corrected second order rates of the oxidation of the Hipips by ferricyanide. In determining if the ionization of a particular amino acid residue is linked to the electron transfer reaction, typically the variation of the reaction rate is followed as a function of pH. With redox proteins such an apparently simple study is complicated by the fact that as the pH is varied the net protein charge varies [32,22]. It is the latter change in net protein charge that can be seriously misleading and mask mechanistically important ionizations [22]. Electron transfer reactions differ from typical enzymatic reactions in that in the former there is no bond making or bond breaking. The consequence of this fact is that the free energy of activation due to bond making-bond breaking is nil, and the free energy contribution from the electrostatic interactions between the reactants (protein-protein or small molecule-protein) becomes significant and observable under conditions of changing ionic strength. It is precisely because of these electrostatic interactions that rate constants need to be electrostatically corrected in order to be able to obtain second order rate constants that do not reflect electrostatic interactions but rather the effect of mechanistically important ionizations [22—24]. In the kinetics (see Table II for results) done in this work, electrostatic effects were effectively partitioned from mechanistically important ionizations, and two pK_a values were revealed at pH $\simeq$ 7 and pH $\simeq$ 10 (see Fig. 7). The $pK_a \simeq 7$ again is that expected for a histidine residue. In earlier work when the rates were not corrected for electrostatic interaction (due to changes in pH and ionic strength) no such pK_a values were observed [8].

Just as importantly, it has been observed that the $E^{0'}$ values of *C. vinosum* [8] and *R. gelatinosa* [33] decreases in going from lower pH to higher pH (i.e. an approximately 30 mV drop in going from pH 5.0 to pH 9.0) with approximate pK_a values of 6.0 and 7.5, respectively. These pK_a values correspond closely to the pK_a values obtained in this work from the pH-absorption difference spectroscopy and pH-kinetic results. In further considering our kinetic results, the Marcus theory of electron transfer [34] predicts that:

$$\log k_{12} = 1/2\{\log k_{11} + \log k_{22} + (\Delta E^0_{12}/0.059)\} \quad (3)$$

where k_{12} is the observed redox rate (cross reaction) between reactants 1 and 2, k_{11} and k_{22} are the self exchange electron transfer rates of the reactants, and ΔE^0_{12} is the difference in the E^0 values of the two reactants. Letting E^0_1 equal the redox potential of ferricyanide, and the $E^{0'}_2$ values equal the redox potentials of the native and diethylpyrocarbonate (DEP)-modified (high pH)

forms of Hipip, then a relative form of the Marcus equation can be easily derived as:

$$\frac{\log k_{12}(\text{DEP modified/high pH})}{\log k_{12}(\text{Native, pH 5})} = \frac{1}{2(0.059)}\{E^0_{\text{Native, pH 5}} - E^0_{\text{DEP/high pH}}\} \quad (4)$$

Now the values of k_{12}(native, pH 9)/k_{12}(native, pH 5) and k_{12}(DEP Hipip)/k_{12}(native, pH 6) can be calculated from the experimental results in Table II and are 1.63 and 1.46. The difference (and the decrease) in redox potential between native Hipip (pH 5 or 6) and the diethylpyrocarbonate or pH 9.0 Hipip necessary to obtain these ratio of redox states can be calculated from Eqn. 4 and are 25 mV and 20 mV, respectively. This range of 20—25 mV represents the decrease in the $E^{0'}$ of Hipip after deprotonation or diethylpyrocarbonate modification and increases the driving force for the ferricyanide oxidation of Hipip, since the difference in $E^{0'}$ values between the two reactants increases. Most importantly this drop in the $E^{0'}$ of Hipip, calculated from the experimental data of this work, compares very well with the drop in the Hipip $E^{0'}$ in going from low pH to higher pH [8,34], thus correlating finally all the observed pK_a values: $E^{0'}$ vs. pH, k_∞ vs. pH, and ΔA (aromatic region) vs. pH.

Destabilization of the reduced state of Hipip is reflected in both the spectroscopic studies, the pH dependence of the E^0 values and their enhanced rate of oxidation by ferricyanide. That the electrostatically corrected rates are valid is shown by the correspondence of the net charge of Hipip (at a given pH) calculated from Eqn. 1 with that net charge of the protein obtained from the cluster charge and primary sequence, Z_{seq} (see Table III). More generally, it is concluded from these studies that it is His-42 that modulates the observed structural and functional differences.

Most critically the diethylpyrocarbonate-modified protein (which was prepared and kinetically examined at pH = 6.0) was indeed faster in its oxidation ($k_\infty = 6.02 \times 10^3\ M^{-1} \cdot s^{-1}$) than the native Hipip and compared well with maximum rate of about $6.7 \times 10^3\ M^{-1} \cdot s^{-1}$ for the deprotonated Hipip (see Fig. 7). Also of interest is that the predicted charge of diethylpyrocarbonate Hipip is —3.7 which compares well with —3.4 for native Hipip at the same pH = 6, thus indicating that few other amino acid residues (e.g., lysines) have been modified. From electrostatic considerations alone, the faster rate for diethylpyrocarbonate Hipip is in opposite directions expected for a protein that has been made slightly more negative in its net charge.

Although somewhat speculative, a more detailed understanding of how deprotonation or diethylpyrocarbonate modification destabilizes the reduced state can be obtained from the X-ray structure studies. Carter and coworkers [4,5] have examined Hipip's X-ray structure and found that it can be divided into two parts of roughly equal size, with their meeting at an 'intramolecular interface'. The $Fe_4S_4^*$ cluster is oriented inside a cavity at the intramolecular interface in such a way that the Fe-S* bonds that undergo the greatest change upon changing redox state are approximately perpendicular to the two halves

of the intramolecular interface. In going from the oxidized to the reduced state the Fe-S* bonds undergo an increase of 2.22Å to 2.38Å [4]. In relating these redox structural changes to the spectroscopy of this work, it would appear that in going from the oxidized to reduced state, the cluster expansion against the surrounding polypeptide increases the hydrophobicity of the environment of the tryptophan(s) and results in the observed red shift in the aromatic region (see Fig. 4). This is equivalent to the idea of Carter et al. [4] of the 'squeeze effect' in which in the reduced state the polypeptide is more tightly wrapped around the cluster. Furthermore, upon either deprotonation or diethylpyrocarbonate modification of His-42, similar red shifted perturbation spectra are observed, and indicate that the polypeptide does indeed wrap itself around the cluster more tightly after these influences. Similarly, in the visible region of the spectrum, deprotonation and diethylpyrocarbonate modification of reduced Hipip result in perturbation spectra (see Fig. 6) that show the cluster to resemble more closely the oxidized state of the protein. This would result from the 'squeeze effect' and results in the destabilization of the reduced state of the protein and this is reflected in the enhanced rate of oxidation of Hipip (see Fig. 7) by ferricyanide. Upon deprotonation or diethylpyrocarbonate modification the polypeptide apparently 'squeezes' the reduced cluster towards the smaller oxidized configuration. However when Hipip is in the oxidized state, there is less influence of the polypeptide on the smaller size cluster as can be seen by the smaller perturbation produced in the visible region of diethylpyrocarbonate-modified oxidized Hipip (see Fig. 4). The 'squeeze effect' as seen spectroscopically would not necessarily require large conformational changes in Hipip. As such these small changes in the size and shape of Hipip do not affect the applicability of Eqn. 1 to both native and modified forms of Hipip.

In considering the mechanism by which deprotonation or diethylpyrocarbonate modification of Hipip causes the 'squeeze effect', we can examine the X-ray crystallographic studies. This work, in contrast to the X-ray crystallographic structure [5], indicates that His-42 is almost certainly not freely extended into the outer solvent, but necessarily interacts with some other portion or amino acid side chain of the polypeptide. One interesting possibility is that His-42 hydrogen bonds to the strictly conserved Asn 45 [19] and upon an increase in pH or diethylpyrocarbonate modification, this interaction is disrupted. The orientation of this hydrogen bond appears to be parallel to the ultimate direction of expansion of the cluster upon reduction. The disruption of this hydrogen bond would then permit the two halves of the intramolecular interface to come closer together, since the cage structure of the cluster site will have been weakened.

In conclusion, kinetic and spectroscopic evidence has been presented and discussed which indicates strongly that the deprotonation of His-42 modulates the structure of the polypeptide and destabilizes the reduced state of the protein and facilitates its oxidation by ferricyanide. Lastly the significance of electrostatically corrected rate constants has been shown. Although the rate changes are not particularly large, it is important to note that Hipip-ferricyanide is a nonphysiological redox couple and that the control of redox kinetics by His-42 could easily prove to be of much greater significance in the biological redox couple, which at this time has not been discovered.

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