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Influence of Surface Charges on Redox Properties in High Potential Iron-Sulfur Proteins

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The pH-dependence of the reduction potential determined through differential pulse voltammetry for the high potential iron sulfur proteins (HiPIP) from *R. globiformis, C. vinosum, R. gelatinosus, E. vacuolata* (I and II), *E. halophila* (I and II) is reported. A decrease in reduction potential with pH is invariably observed in the pH range where deprotonation of the imidazolium nitrogen of histidine residue(s) occurs. No pH dependence is observed for the only protein lacking histidines. It appears that surface charges like the His imidazolium groups are capable of influencing the reduction potential despite the known quencing of the electrostatic interactions due to solvent effects.

There is an increasing interest in understanding the determinants of the oxidation-reduction properties of electron-transport proteins and redox proteins in general. Theoretical models are being developed to account for electrostatic interactions, electric dipole effects, hydrogen-bonding, Van der Waals interactions, electronic effects, etc. Encouraging successes have been achieved (1-7). However, the various models still suffer from the scarcity of adequate test systems. Ideally, for each of the various interactions, a series of data would be needed in which only one interaction is modulated whereas the contributions from all other effects is

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either negligible or constant within the series. Reliable electrochemical data can nowadays be obtained on metalloproteins through a variety of techniques (8).

High potential iron-sulfur proteins constitute a class of iron-sulfur proteins characterized by positive E° values spanning a ~400 mV range (9, 10). They all contain a Fe_4S_4 cluster, with total charge +3 and +2 in the oxidized and reduced states, respectively. The X-ray structures of the HiPIPs from *C. vinosum*, *E. halophila* (iso I), *R. tenuis* and *E. vacuolata* II are available (11-14); an MD model of the structure of HiPIP from *E. halophila* (iso II) is also available (15). The tertiary structures are very similar. In particular, the clusters are coordinated by four conserved cysteines, and the coordinated sulfur atoms are involved in five conserved hydrogen-bonds with peptide NH protons (16). These features are maintained in both the oxidation states (11-14). The coordination geometries around each iron are also very similar. On the other hand, the number of charged residues in the different proteins varies sizably from one protein to another, the total protein charge ranging between -14 and +4 (Table 1). Preliminary data indicate that a correlation may exists between the number and distribution of charged residues in the various protein and their reduction potentials (6, 17).

With this in mind, we have measured the pH-dependence of the reduction potentials, E°, of seven different HiPIP's. These data add to the existing pH profiles for other two proteins of the series (18), providing a total of nine different data sets in an homogeneous series of compounds. We find that around neutrality there is a significant decrease of E° with increasing pH, that is entirely due to the deprotonation of histidines; therefore, the change of redox potential in this region with increasing pH can be uniquely related to the disappearance of the electrostatic effect of the positive charge of the imidazole rings. This finding has implications for the theoretical treatments of the electrostatic effects on the surface charges in proteins.

Materials and Methods

Proteins were isolated as described elsewhere (19-21). E° values were determined by differential pulse voltammetry. A freshly polished pyrolytic graphite disc (edge plane) was used as working electrode, a Pt sheet as counter, and a saturated calomel electrode (SCE) as the reference electrode (22). However, all the potentials reported in this paper are referred to the normal hydrogen electrode (NHE). 0.5 M NaCl was used as base electrolyte. The reversibility of the electrochemical process was evaluated from the width at the half-peak ($91/\alpha$ mV). In all cases the process is quasi-reversible ($\alpha = 0.8 \div 0.9$). The $E_{1/2}$ values were obtained by applying the Parry-Osteryoung relationship that relates the peak potential to the half-wave potential $E_{1/2}$ for a given pulse amplitude ΔE. The potentials were calibrated against the MeV^{2+}/MeV couple (E° = -0.446 V vs. NHE). A cell for small volume samples (about 0.5 cm^3) working under argon atmosphere at 25±0.1°C was used. Unbuffered protein solutions were freshly

prepared before use and their concentration, varying from 0.1 to 0.3 mM, was checked spectrophotometrically. The pH was changed by adding small amounts of concentrated NaOH or HCl under fast stirring. Measurements were carried out down to the lowest pH value that preserved sample integrity. In all cases measurements could not be performed below pH 4.

To evaluate electrostatic interactions, the individual distances between the charged histidines and the cluster had to be estimated. For the four proteins from *C. vinosum*, *E. vacuolata* iso-I and *E. halophila* iso-I and iso-II, for which X-ray or MD structures are available, the distances have been estimated directly. The distances are taken from the center of the aromatic ring to the center of the cluster. For all the other proteins, conserved histidines have been assumed to maintain the same coordinates as in the X-ray structure of the protein with the highest sequence homology, whereas non conserved histidines were substituted in the proper sequence position and the side chain was oriented in such a way as to minimize Van der Waals contacts and to form hydrogen bonds when possible. The starting structure was that of *C. vinosum* HiPIP for *T. pfennigii*, *R. gelatinosus* and *P. halodenitricans* HiPIP's, and that of *E. halophila* HiPIP (iso-I) for *E. vacuolata* (iso-I and iso-II), and *R. globiformis* HiPIP's.

Results and Discussion

The E° values of the HiPIP's from *R. globiformis*, *C. vinosum*, *R. gelatinosus*, *E. vacuolata* (iso-I and iso-II), *E. halophila* (iso-I and iso-II) as a function of pH are reported in Figure 1, together with those previously obtained for HiPIP's from *T. pfennigii* and *P. halodenitricans* (18). In all cases but one a sizable decrease in reduction potential with increasing pH is obtained. Such a decrease is in qualitative agreement with loss of protons, hence with an increase of the overall negative charge (or decrease of the positive charge for *R. gelatinosus)* of the protein. The one HiPIP that shows E° values which are virtually pH independent is that from *R. globiformis*, which contains no histidines. The decrease in reduction potential per pH unit, which is invariably much lower than 59 mV, and the sigmoid shape of the curves, indicate that the redox equilibrium of the cluster does not involve a net uptake or release of protons (2, 23). Acid-base groups may influence the electron exchange of the polymetallic center through eletrostatic interactions. Proteins from *T. pfennigii* and *P. halodenitricans* show an additional equilibrium with pK_a values of 8.0 and 9.3, respectively. The latter may be too high to be attributed to histidine residues.

The E° values for the proteins from *C. vinosum*, *R. gelatinosus*, *E. vacuolata* (iso-II), *E. halophila* (iso-I) and *E. halophila* (iso-II) do not reach a plateau at pH values around 5, but increase further with decreasing pH. This increase in E° can be reasonably assigned to the acid base equilibria of Glu and Asp residues (Table 1) which are known to affect the cluster, as previously shown by ^1H NMR titrations of *C. gracile* HiPIP (24). We discard the possibility that such an E° increase arises from a partial protein unfolding causing disruption of the hydrogen

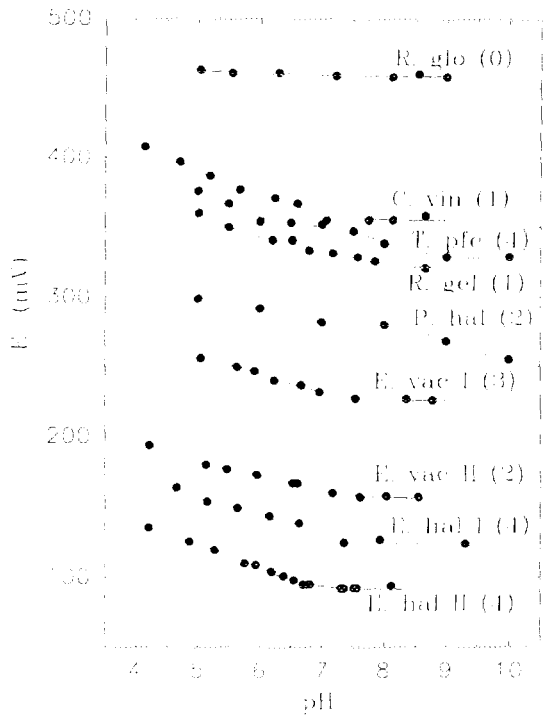

Figure 1. pH-dependence of the reduction potential of various HiPIPs. The number of histidine residues in each protein is shown in parentheses. Also reported are the best fit curves to equation 1. T=25°C.

bonding network that stabilizes the 3+ state of the cluster, since ^{1}H NMR measurements indicate that the overall protein structure is largely conserved well below pH 5 (24). In multi-histidine proteins the various equilibria may influence each other. The individual microscopic pK_a values as well as the extent of individual contributions to the overall ΔE° cannot be unambiguously determined within the present approach. Apparent pK_a values for carboxylate and histidine residues, which simply refer to the average overall influence of the above two classes of ionizing groups on the reduction potential, can be estimated by fitting the data of Fig.1 with the following two-proton equilibria equation (1):

$$E^\circ = E^\circ_a - 0.06 \cdot \log \frac{[H^+]^2 + [H^+] \cdot K_{o1} + K_{o1} \cdot K_{o2}}{[H^+]^2 + [H^+] \cdot K_{r1} + K_{r1} \cdot K_{r2}} \qquad 1)$$

The values are reported in Table 1. E°_a is the limit E° values for the protein containing Asp, Glu and His residues in the fully protonated form, and K_1 and K_2 correspond to the apparent dissociation constant for the carboxylate residues and for the histidine residues, respectively. Indexes r and o refer to the reduced and oxidized proteins. Very similar values of E°_a and equilibrium constants for histidine

Table 1. Reduction potential at neutral pH and apparent pK of histidine deprotonation for different HiPIPs [a]

HiPIP	E° (mV)	pK_r	pK_0	n° His	n°Asp + Glu	total charge[b]
R. globiformis	461	---	---	0	8	-1
C. vinosum	355	6.6±0.3	6.2±0.3	1	9	-3
T. pfennigii	350[c]	5.5±0.2[d]	4.4±0.4[d]	4	11	-8
R. gelatinosus	333	7.5±0.2	7.2±0.2	1	5	+4
P. halodenitricans	282[c]	6.3±0.2[d]	6.0±0.2[d]	2	11	-11
E. vacuolata iso-I	232	6.5±0.1	6.0±0.1	3	8	-4
E. vacuolata iso-II	162	6.7±0.1	6.4±0.1	2	10	-7
E. halophila iso-I	133	6.9±0.2	6.4±0.3	4	14	-11
E. halophila iso-II	93	6.2±0.1	5.7±0.1	4	16	-14

a) pK_r and pK_0 values refer to the reduced and oxidized proteins. The estimated error on the reduction potentials is ± 3 mV. pK values are given with the absolute error obtained from the fitting to equation 1.
b) Referred to the oxidized protein; histidines are considered neutral.
c) from ref. 15.
d) obtained from fitting of data of ref. 15.

deprotonation are obtained by using a one-proton equilibrium equation and neglecting in the calculations some of the low-pH E° values. For the proteins from *T. pfennigii* and *P. halodenitricans*, which also show two titration steps, the same equation was applied, but in this case histidine deprotonation is clearly related to the K_{r1} and K_{o1} values, K_{r2} and K_{o2} being exceedingly high, as noted above. Finally, the E° data for *E. vacuolata* (iso-I) HiPIP were satisfactorily fitted with a one-proton equilibrium equation. The pK values for histidine deprotonation for *C. vinosum* and *R. gelatinosus* HiPIP, which are meaningful estimates of indifidual pK_as since these proteins contain only one histidine, are in the same range as those determined by ^1H NMR (25). The pK values for *T. pfennigii* and *R. gelatinosus* HiPIP, although somewhat different from the others, are still inside the pK_a range for the deprotonation of the imidazolium nitrogen of histidine (26).

There is no obvious relation between the number of histidines and the extent of the decrease of the reduction potential with pH. This is not surprising because the electrostatic interaction must be a function of the location of each particular histidine with respect to the cluster. We have tried to analyze the data on simple electrostatic grounds, once evaluated the individual histidine-cluster distances from X-ray or MD structures, without attempting to singled out the change in reduction potential due to each histidine deprotonation. There is only a rough correlation between the total $\Delta E°$ and the number of histidines in each protein, even when the

latter are weighted by the reciprocal of their individual distances from the cluster. Inter-histidine interactions make the observed ΔE° not simply given by the sum of the individual contributions. Therefore no information can be extracted from this simple approach on the variability of the dielectric properties of the protein medium. Qualitatively, the decrease in reduction potential of about 20 mV due to the loss of a proton in the one-histidine HiPIPs from *C. vinosum* and *R. gelatinosus* indicates the sensitivity of the thermodynamics of the cluster to electrostatic interactions, and hence points out the relevance of the electric charges as possible determinant of the variability of the redox potential in this class of proteins (6, 17). In this respect, the fact that in the series the total protein charge spans from +4 to -14 might, by itself, account for the about 400 mV range of the E° values.

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References

1. Moore, G. R., and Pettigrew, G. W. (1990) Cytochromes c: Evolutionary, Structural and Physicochemical Aspects, Ch. 7, Springer Verlag, Berlin.
2. Armstrong, F. A. (1992) In Advances in Inorganic Chemistry (R. Cammack, Ed.) Vol. 38, pp. 117-163, Academic Press, San Diego.
3. Churg, A. K., and Warshel, A. (1986) Biochemistry 25, 1675-1681.
4. Rogers, N. K., and Moore, G. R. (1988) FEBS Lett. 228, 69-73.
5. Langen, R., Jensen, G. M., Jacob, U., Stephens, P. J., and Warshel, A. (1992) J. Biol. Chem. 267, 25625-25627.
6. Rees, D. C. (1985) Proc. Natl. Acad. Sci. USA 82, 3082-3085.
7. Backes, G., Mino, Y., Loehr, T.M., Meyer, T. E., Cusanovich, M. A., Sweeney, W. V., Adman, E. T., and Sanders-Loehr, J. (1991) J. Am. Chem. Soc. 113, 2055-2064.
8. Armstrong, F. A. (1990) Struct. Bonding, 72, 137-221.
9. Cammack, R. (1987) In Iron-Sulfur Protein Research; (H. Matsubara, Y. Katsube, K. Wada, Eds), p 40, Japan Scientific Societies Press / Spriger Verlag, Berlin.
10. Evans, M. C. W. (1982) In Iron-Sulfur Proteins (T. G. Spiro, Ed.), Ch. 6, Wiley, New York.
11. Carter, C. W., Jr., Kraut, J., Freer, S. T., Xuong, N.-H., Alden, R. A., and Bartsch, R.G. (1974) J. Biol. Chem. 249, 4212-4225.
12. Carter, C. W., Jr., Kraut, J., Freer, S. T., and Alden, R. A. (1974) J. Biol. Chem. 249, 6339-6346.
13. Breiter, D. R., Meyer, T. E., Rayment, I., and Holden, H. M. (1991) J. Biol. Chem. 266, 18660-18667.
14. Rayment, I., Wesemberg, G., Meyer, T. E., Cusanovich, M. A.. and Holden, H. M. (1992) J. Mol. Biol. 228, 672-686.

15. Banci, L., Bertini, I., Capozzi, F., Carloni, P., Ciurli, S., Luchinat, C., and Piccioli, M. (1993) J. Am. Chem. Soc. 115, 3431-3440.
16. Backes, G., Mino, Y., Loehr, T. M., Meyer, T. E., Cusanovich, M. A., Sweeney, W. V., Adman, E. T., and Sanders-Loehr, J. (1991) J. Am. Chem. Soc. 113, 2055-2064.
17. Banci, L., Bertini, I., Capozzi, F., Ciurli, S., Gori Savellini, G., and Luchinat, C. (1993) Proceeding of the first Siena-Kyoto Meeting, Kyoto.
18. Mizrahi, I. A., Meyer, T. E., Cusanovich, .M. A. (1980) Biochemistry 19, 4727-4733.
19. Bartsch, R. G. (1978) Methods Enzymol. 53, 329-340.
20. Kusche, W. H., and Truper, H. G. (1984) Arch. Microbiol. 137, 266-271.
21. Bertini, I., Capozzi, F., Luchinat, C., and Piccioli, M. (1993) Eur. J. Biochem. 212, 69-78.
22. Borsari, M., Sola, M., Cowan, J. A. (1991) Bioelectrochem. Bioenerg. 26, 123-129.
23. Tanaka, K., Moriya, M., and Tanaka, T. (1986) Inorg. Chem. 25, 835-838.
24. Sola, M., Cowan, J. A., and Gray, H. B. (1989) Biochemistry 28, 5261-5268.
25. Nettesheim, D. G., Meyer, T. E., Feinberg, B. A., and Otvos, J. D. (1983) J. Biol. Chem. 258, 8235-8239.
26. Valcour, A. A., and Woodworth, R. C. (1987) Biochemistry 26, 3120-3125.