A Comparison of Fe_4S_4^* Clusters in High-Potential Iron Protein and in Ferredoxin

(protein structure refinement/electrode potentials)

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Communicated by Martin D. Kamen, September 25, 1972

ABSTRACT The structures of both oxidized (HP_{ox}) and reduced (HP_{red}) high-potential iron protein and of oxidized ferredoxin (Fd_{ox}) have been partially refined at 2.0-Å resolution by methods similar to those applied to the protein rubredoxin [Watenpaugh, K. D., Sieker, L. C., Herriott, J. R. & Jensen, L. H. (1971) *Cold Spring Harbor Symp. Quant. Biol.* 36, 359-367]. Average bond lengths and angles in the HP_{red} and Fd_{ox} Fe_4S_4^* clusters are the same to within the root-mean square (rms) deviation of each mean value. A preliminary comparison of the two HiPIP oxidation states indicates that the HP_{ox} cluster is geometrically similar to the HP_{red} cluster, but that it is smaller by 0.1-0.2 Å in certain dimensions. The HiPIP and ferredoxin cluster geometry is also nearly identical to that reported recently for a synthetic analog [Herskovitz, T., Averill, B. A., Holm, R. A., Ibers, J. A., Phillips, W. D. & Weiher, J. F. (1972) *Proc. Nat. Acad. Sci. USA* 69, 2437-2441]. An apparent paradox presented by the large difference between the HiPIP and ferredoxin electrode potentials can be resolved by the assumption that the Fe_4S_4^* cluster has not two but *three* oxidation states. The fully oxidized (HP_{ox}) and fully reduced (Fd_{red}) clusters are paramagnetic, and the intermediate state is spin-paired [Tsibris, J. C. M. and Woody, R. W. (1970) *Coord. Chem. Rev.* 5, 417-458]. This hypothesis is supported by structural and spectroscopic evidence that the "paired-spin" state exists in both HP_{red} and Fd_{ox} .

An unusual iron-sulfur prosthetic group has emerged from examination of the crystal structure of high-potential iron protein (HiPIP) from *Chromatium* and of *Peptococcus aerogenes* ferredoxin. The iron-sulfur (Fe_4S_4^* ; S^* indicates inorganic sulfur) cluster in HiPIP (1-3) may be simply described as an inorganic cubane compound bound to the protein by bonds from four cysteinyl sulfur atoms to the four iron atoms. The four iron and four inorganic sulfur atoms form approximately regular, interpenetrating, and concentric tetrahedra (ref. 3, Fig. 2). Two similar clusters have now been identified in the bacterial-type ferredoxin from *P. aerogenes* (4). Together, these two crystallographic studies suggest that this moiety is a prosthetic group of fundamental significance.

Abbreviations: HiPIP, high-potential iron protein; HP_{ox} , oxidized HiPIP; HP_{red} , reduced HiPIP; Fd_{ox} , oxidized ferredoxin; S^* , inorganic sulfur; S_γ , cysteinyl sulfur. $R = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$, crystallographic R factor.

^{††} This work is taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of California, San Diego.

We report here recent crystallographic results that show that the reduced HiPIP and oxidized ferredoxin clusters are indeed geometrically indistinguishable. In addition, we propose a novel explanation for the large difference (0.75 V) between the electrode potentials, E_o' , of the two proteins.

METHODS

Structures of both oxidized and reduced HiPIP (HP_{ox} and HP_{red} , respectively) and of oxidized ferredoxin (Fd_{ox}) have been partially refined at 2.0-Å resolution by ($F_o - F_c$) synthesis methods similar to those applied to rubredoxin (5). In addition, the 12 atoms of the iron-sulfur clusters in each case (HP_{ox} , HP_{red} , and Fd_{ox}) have been refined by least-squares methods. Because crystallographic refinement procedures have not been widely applied to protein structures, it is appropriate to justify our belief that these procedures are effective. First of all, present crystallographic R factors of 0.17, 0.19, and 0.34 for HP_{ox} , HP_{red} , and Fd_{ox} , respectively, compare favorably with unrefined R factors of about 0.43 each for both proteins. Secondly, in the refinement of HiPIP this decrease in the R factor was accompanied by increased clarity in ($F_o - F_c$) difference Fourier maps computed for the purpose of displaying just the eight sulfur atoms. A similar improvement in clarity has been observed for the whole ΔF map for Fd_{ox} . Third, as indicated by the root-mean square (rms) deviations of bond lengths in Table 1, the iron-sulfur distances of the refined structure are now more self-consistent. Thus in HiPIP, the rms deviation for iron-inorganic sulfur distances ($\text{Fe}-\text{S}^*$) and for iron-cysteinyl sulfur distances ($\text{Fe}-\text{S}_\gamma$) fell to 0.08 and 0.02 from 0.23 and 0.12, respectively. Finally, the $\text{Fe}-\text{S}_\gamma$ distances in HiPIP now average 2.20 Å, in contrast to the unreasonably low value of 2.01 Å reported for the unrefined structure (3). Thus, the reduction in R factors, together with the observed improvement in ($F_o - F_c$) difference maps and in iron-sulfur stereochemistry, indicate that crystallographic refinement has indeed produced more reliable structures.

RESULTS

We have calculated mean values for chemically equivalent bond lengths and bond angles in the HP_{ox} , HP_{red} , and Fd_{ox} clusters. Results given in Table 1, together with the range, rms deviation, and number of observations contributing

to each mean value, demonstrate convincingly the identity of HiPIP and ferredoxin prosthetic groups. Thus, average bond lengths and angles in the reduced HiPIP and oxidized ferredoxin clusters are the same to within the rms deviation of each mean value. As we have already indicated, the rms deviation for a given type of bond in HiPIP is small (0.02–0.09 Å), so it may be concluded that the iron and both sulfur tetrahedra are nearly regular in HP_{ox} and HP_{red}. The ferredoxin clusters are not yet refined sufficiently to justify the same conclusion for Fd_{ox}, but we note that the rms deviations are approximately equal to those observed for HiPIP at a comparable stage in the refinement. We anticipate that they will improve with further refinement.

Distortions of the cluster geometry resulting from asymmetry of the polypeptide environment must be relatively small. However, the uncertainty of each bond length is still too great to justify comparison of individual interatomic distances and angles. Therefore, the distinct possibility remains that the HP_{red} and Fd_{ox} clusters are affected differently by their protein environments and that these differences are still too subtle to identify crystallographically at our present resolution.

The following interpretation of the data in Table 1 is of heuristic value in rationalizing the geometry of the Fe₄S₄* cluster. On the one hand, because X–S–X bond angles between 90° and 105° are normally observed for sulfides (6), one might expect the cluster's Fe–S*–Fe angles to be in this neighborhood. On the other hand, since each iron atom is surrounded by four sulfur atoms, one might expect S*–Fe–S* angles close to the tetrahedral angle 109° 28'. However, we note that these angular requirements are mutually incompatible on simple geometrical grounds. Thus, in the first case, 90° Fe–S*–Fe angles could occur only if the Fe₄S₄* moiety were a cube, which would of course also require 90° S*–Fe–S* angles at each iron atom. In the second case, uniformly tetrahedral S*–Fe–S* angles would require 66° Fe–S*–Fe angles at each inorganic sulfur atom. This hypothetical limiting structure would closely approximate a regular stellated tetrahedron. In fact we find that the Fe₄S₄* cluster geometry actually observed is a compromise between the conflicting architectural demands of these two hypothetical limiting structures. More precisely, instead of being either 66° or 90°, the Fe–S*–Fe angles average 75°; and instead of being either 90° or 109° 28', S*–Fe–S* angles average 103°. Furthermore, we note that the three S*–Fe–Sγ angles to each cysteinyl sulfur atom average 116°, so that the average of the six S–Fe–S angles around each iron atom is 109°. To what extent does the Fe₄S₄* cluster resemble either of the two hypothetical limiting structures? We can answer this question by comparing the ratios of the mean Fe–S* distance to the mean Fe–Fe distance in the Fe₄S₄* clusters with that for a cube (0.71) and for the stellated tetrahedron (0.92). In fact, the ratio is found to be intermediate between these limits: 0.83 for HP_{ox} and HP_{red}, and 0.81 for Fd_{ox}. We believe that these two values are not significantly different.

Although the HP_{ox}, HP_{red}, and Fd_{ox} clusters are similar in that they have the same proportions, only the HP_{red} and Fd_{ox} clusters are geometrically congruent in the sense that they also have the same average iron–iron distance. Thus, the mean iron–iron distances of HP_{ox} (2.72 Å) and of HP_{red} (2.81 Å) differ by 0.09 Å, or by twice the rms deviation of

TABLE 1. Mean values for equivalent bond lengths and angles in the HiPIP and ferredoxin Fe₄S₄* clusters

		HP _{ox}	HP _{red}	Fd _{ox}
<i>Bond lengths (Å)</i>				
Fe–Fe	Mean	2.72	2.81	2.85
	range	2.68–2.78	2.74–2.87	2.65–3.04
	rms dev	0.04	0.04 _s	0.10
	n	6	6	12
Fe–S*	Mean	2.26	2.32	2.30
	range	2.10–2.39	2.18–2.45	1.93–2.67
	rms dev	0.08	0.09	0.23
	n	12	12	24
Fe–Sγ	Mean	2.20	2.22	2.19
	range	2.17–2.22	2.19–2.26	1.90–2.44
	rms dev	0.02	0.03	0.17
	n	4	4	8
<i>Bond angles (°)</i>				
Fe–S*–Fe	Mean	74	76	78
	range	72–76	72–80	66–91
	rms dev	1.3	2.4	5.8
	n	12	12	24
S*–Fe–S*	Mean	104	104	101
	range	101–109	99–107	88–116
	rms dev	2.4	2.6	8.4
	n	12	12	24
S*–Fe–Sγ	Mean	115	116	116
	range	107–120	106–126	95–140
	rms dev	4.9	5.3	9.5
	n	12	12	24

either mean. This difference between HP_{ox} and HP_{red} is confirmed by the oxidized-minus-reduced difference Fourier map. A preliminary interpretation of the difference, consistent both with the difference map and with the refined coordinates, is that pairs of opposite Fe₂S₂* faces of the cluster move relative to each other upon oxidation. Two pairs of faces move closer together by about 0.2 Å and 0.1 Å and one pair moves further apart by 0.1 Å. In short, the oxidized cluster is smaller than the reduced cluster. We hasten to add that the HiPIP polypeptide-chain conformation is unaffected by this structural rearrangement. We have not yet compared the structures of oxidized and reduced ferredoxin, but it is unlikely that ferredoxin undergoes a significant conformational change either.

Herskovitz *et al.* (7) have reported recently the synthesis and structure of a compound containing an Fe₄S₄* core with a mercaptide sulfur (R = SCH₂C₆H₅) bonded to each iron atom. Average dimensions given in Table 1 for the HP_{red}, HP_{ox}, and Fd_{ox} clusters agree in surprising detail with those of this analog. Specifically, the mean refined Fe–Fe distance of 2.81 Å for HP_{red} compares more favorably with 2.75 Å for the analog than did the corresponding value of 3.06 Å reported previously for the unrefined structure (3). The mean iron–sulfur distances (Fe–S*, Fe–Sγ) of 2.32 Å and 2.22 Å for HP_{red} are also close to the values 2.29 Å and 2.25 Å for the analog. Mean bond angles agree even more closely. It is particularly notable that both the synthetic and the protein-bound clusters have two sets of average S–Fe–S angles (S*–Fe–Sγ, S*–Fe–S*), which are 116° and 104° in HP_{red} and 115° and 104° in the analog. Finally, we note that the symmetry of the analog is actually tetragonal (point group *D*_{2d}–42*m*) and is only approximately tetrahedral (point group

Fig. 1. The three-state hypothesis for relative oxidation states of the Fe_4S_4^* cluster in iron-sulfur proteins. The "paired-spin" state, "C", occurs in both reduced HiPIP and oxidized ferredoxin. It is experimentally indistinguishable in the two proteins.

$T_d\bar{4}3m$), because it has two distinct sets of Fe-S* distances: one, a set of eight, which averages 2.31 Å; the other, a set of four parallel to the $\bar{4}$ axis, which averages 2.24 Å. In this regard, it may be more than coincidental that an orientation of the HP_{red} cluster may be chosen so that corresponding sets of eight and four Fe-S* distances average 2.35 Å and 2.25 Å, respectively.

DISCUSSION

As we mentioned at the outset, there is a large (0.75 V) difference between the electrode potentials of HiPIP ($E_0' = +0.35$ V) and ferredoxin ($E_0' = -0.40$ V). Our finding that both proteins contain the same prosthetic group, i.e., the Fe_4S_4^* cluster, thus presents what at first appears to be a paradox. A potential difference of 0.75 V implies that a free energy change, ΔG_0 , of -17.3 Cal/mol accompanies electron transfer to the oxidized HiPIP cluster from the reduced ferredoxin cluster: $\text{HP}_{\text{ox}} + \text{Fd}_{\text{red}} \rightleftharpoons \text{HP}_{\text{red}} + \text{Fd}_{\text{ox}}$ (I). If it is assumed (incorrectly, as we believe) that only two oxidation states are accessible to the Fe_4S_4^* cluster, then the net effect of reaction (I) would be simply to relocate one oxidized cluster and one reduced cluster into different protein environments. But it is doubtful, on the one hand, that any protein is rigid enough to impose the necessary stereochemical constraints on the cluster to account for a substantial fraction of the 17.3 Cal/mol free-energy change. On the other hand, if a change in the oxidation state of the cluster were accompanied by a large conformational change, involving a large ΔG_0 , we would expect to observe this change crystallographically. As mentioned above, we observe no conformational change for the oxidation of HiPIP, and there is no chemical evidence leading us to expect one in ferredoxin. The apparent paradox, then,

is that 0.75 V is too big a potential difference to achieve by manipulating the protein environments of two oxidation states of the same prosthetic group.

The difficulty is easily resolved, however, if we assume that not two, but *three* oxidation states are available to the Fe_4S_4^* cluster, and that HiPIP and ferredoxin use, respectively, the oxidation and reduction of what is essentially the same species, "C". This proposal is illustrated in Fig. 1. The two successive electron-transfer reactions must have inherently different electrode potentials, which may account for essentially all the potential difference between the physiological reactions of HiPIP and ferredoxin.

The "three-state" hypothesis rests on an impressive base of evidence that the clusters in reduced HiPIP and oxidized ferredoxin, both of which should contain state "C", as indicated by the arrows in Fig. 1, are experimentally indistinguishable and must, therefore, have the same oxidation state (7). We have here shown by x-ray crystallography that the clusters are the same in both HP_{red} and Fd_{ox} within the present experimental error. Spectroscopic and magnetic characteristics of these two "C" states have been compared by Carter (9) and have also been presented in detail by Herskovitz *et al.* (7). They are therefore summarized only briefly here.

First, visible absorption spectra of HP_{red} and of Fd_{ox} have maxima at 390 nm of comparable intensity ($\epsilon_{390} = 4[\text{mM}(\text{Fe})]^{-1}$, and $3.8[\text{mM}(\text{Fe})]^{-1}$). Second, the "C" state is essentially nonmagnetic in both proteins (10). We have accordingly designated it the "paired-spin" state in Fig. 1. Third, Phillips and coworkers (11, 12) have found that a residual paramagnetism exists in both HP_{red} and Fd_{ox} that is sufficient to shift the magnetic resonances of the cysteine β -methyl protons to lower fields by about 13.5 ppm. The similar position and temperature dependence of these contact shifts in HP_{red} and Fd_{ox} provide remarkably sensitive evidence that the magnetic properties of these two "paired-spin" states are identical.

The two paramagnetic oxidation states "C+" and "C-" are identified in Fig. 1 with their more familiar spectroscopic properties. The "oxidized" state, "C+", (HP_{ox}) is paramagnetic with a susceptibility compatible with a single unpaired spin, $S = 1/2$, and has an electron paramagnetic resonance at $g = 2.04$ (10). The "reduced" state, "C-", (Fd_{red}) also contains a single unpaired spin and displays electron paramagnetic resonance at $g = 1.94$ (10). We might point out as a concluding comment on the "three-state" hypothesis that other biological systems are also known to possess three or more stable oxidation states. For example, the magnesium tetrapyrrolic chelate, chlorophyll, has three oxidation states—radical half-oxidized, paired-spin, and radical half-reduced—separated by a large potential difference, in strict analogy with the Fe_4S_4^* cluster (8).

We must now direct attention to fundamental questions regarding the interaction of the polypeptide with the cluster. As we have indicated, the structures of "C+" (in HP_{ox}) and "C" (in HP_{red}) both appear to differ only in the bond lengths between pairs of opposite Fe_2S_2^* faces. Unfortunately, the structure of reduced ferredoxin is still unknown, and we therefore cannot characterize the geometry of the reduced cluster, "C-". Nevertheless, we expect from our crystallographic comparison of HP_{ox} and HP_{red} that the three oxidation states of the cluster will differ only by a few tenths of an Angstrom

in their absolute size, "C⁺" being the smallest and "C⁻" being the largest.

If we consider the fact that only small structural rearrangements occur when the HiPIP cluster changes oxidation states, together with our conclusion that the "paired-spin" states of the Fe_4S_4^* cluster are experimentally indistinguishable at present, it is indeed curious that only one of the two possible equilibria accessible to the cluster has been observed in each protein. Specifically, why has not reduction of the cluster to the fully reduced state "C⁻" been observed in HiPIP; conversely, why has not oxidation of the cluster to the oxidized state "C⁺" been observed in ferredoxin? There are, of course, differences between the protein environments of the HiPIP and ferredoxin clusters. Notably, although both polypeptides surround the Fe_4S_4^* cluster with nonpolar amino-acid side chains, the two ferredoxin clusters are more accessible to solvent. However, it is unclear to us how this difference can account for the fact that these equilibria have not yet been observed. Thus, on the basis of the available data we can only speculate. Perhaps, on the one hand, a systematic search for these two postulated equilibria will reveal their existence at electrode potentials at the two extremes of the physiological range, as we have implied in Fig. 1. On the other hand, it is also possible that the change in either the size or the formal charge of the cluster may simply render the unobserved HiPIP-(C⁻) and ferredoxin-(C⁺) unstable, and lead to unfolding of the protein, and hence, to decomposition of the cluster.

As we have already pointed out, the E_0' values for proteins using very similar electron-transfer sites would be expected to vary only over a narrow range, which we have indicated by slashed lines on the potential scale in Fig. 1. Indeed, potentials of different bacterial-type ferredoxins vary by only about 0.1 V. Typical of this range are the values $E_0' = -0.40$ V and $E_0' = -0.49$ V for the *Clostridium* and *Chromatium* ferredoxins, respectively (13). Thus, we suggest that the polypeptide configuration probably exerts a subtle, rather than a dramatic, influence on the thermodynamic properties of the cluster. It now appears from the work of Herskovitz *et al.* (7) that the geometry and the essential spectroscopic, magnetic, and thermodynamic properties of the Fe_4S_4^* clusters in HP_{red} and Fd_{ox} are surprisingly similar to those of a synthetic analog. We therefore conclude that the perturbations imposed on the cluster by the protein are probably subtle in most other respects as well.

We thank R. H. Holm, J. A. Ibers, and W. D. Phillips for a manuscript communicated before publication. This work was supported at the University of California, San Diego by research grants from the National Institutes of Health (GM 10928, GM 16717) and the National Science Foundation (GB 30828X, GB 23054) and by Public Health Service Research Career Development Awards to R. A. A. and S. T. F. from the National Institute of General Medical Sciences (GM 15401, GM 70-140). At the University of Washington this work was supported by a research grant from the National Institutes of Health (GM 13366).

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