New Stereochemical Analogies between Iron-Sulfur Electron Transport Proteins*

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Active sites of Chromatium high potential iron protein (HiPIP) and Pseudomonas aerogenes ferredoxin can be brought into equivalent orientations by assuming that their $\text{Fe}_2\text{S}_2\text{Sy}_4$ clusters have the effective symmetry of the non-axial molecular point group $C_p$. Previously undetected analogies between the two proteins emerge as a result of selecting a common orientation in this manner. Polypeptide segments connecting Cys 46 to Cys 63 in HiPIP and Cys 18 to Cys 35 in ferredoxin are analogous in the sense that they are the same length, they connect equivalent cysteinyl sulfur atoms, and they have similar, twisted antiparallel $\beta$ conformations. Tyrosine residues 19 (HiPIP) and 2 (ferredoxin) are analogous in the sense that they interact closely with equivalent inorganic sulfur atoms.

To a good approximation, interactions with the polypeptide backbone and with tyrosine side chains in the two proteins place their $\text{Fe}_2\text{S}_2\text{Sy}_4$ moieties into diastereomeric environments, which would be expected to induce different physical and chemical behavior. Circular dichroism spectra of native and super reducible HiPIP (Cammaack, R. (1973) Biochem. Biophys. Res. Commun. 54, 548–554) suggest that this relationship can help to explain the contrasting oxidation reduction properties of the two proteins.

From x-ray crystal structures of Chromatium high potential iron protein (1–4) and Pseudomonas aerogenes ferredoxin (5–7) it is now known that these two electron-transport proteins share a common prosthetic group, whose structure appears in Fig. 1. This tetranuclear, cubane-like complex contains four equivalent cysteinyl sulfur atoms, and it is bound to the peptide backbone and with tyrosine side chains in the two proteins. The question persists: How do they do it? One suspects that such an obvious difference in catalytic activities ought to be related, at least in the first approximation, to structural analogies and contrasts between their active sites.

Different enzymes often use similar stereochemical relationships within their active sites to catalyze the same chemical reaction. X-ray crystallography has afforded comparisons between active site geometries for several sets of analogous, but evolutionarily distinct, enzymes (14, 15). Surprising similarities in the catalytic configurations of such pairs of enzymes emerge when their polypeptide chains are superimposed about a common substrate orientation. These comparisons provide direct insight into catalytic mechanisms by suggesting detailed active site models. Models, in turn, motivate explicitly targeted experiments. My purpose here is to describe struc-
Experimental Procedures

Atomic Coordinates – Atomic coordinates for reduced and oxidized HiPIP and for oxidized ferredoxin were provided by Professors Joseph Kraut, University of California, San Diego, and Lyle H. Jensen, University of Washington. These models had been subjected to crystallographic refinement to 2.0 Å data sets by nearly identical methods (3, 7). Stereoechemical constraints on polypeptide geometry were imposed at intervals alternating with cycles of difference synthesis during early stages, and of analytical differential synthesis during later stages of refinement. This procedure would be expected to minimize the extent to which errors in polypeptide synthesis near the Fe&*S4 atoms might influence cluster geometries which, in contrast, were unconstrained. Coordinates of the 12 cluster atoms were further adjusted by least squares refinement using a constant contribution from remaining protein atoms. Crystallographic R factors of 0.106, 0.160, and 0.106 for the final, unconstraineded models of oxidized and reduced HiPIP (3) and oxidized ferredoxin (7) compare favorably with values obtained for other protein structures refined to comparable resolution limits.

Estimated standard deviations obtained from the inverse matrix after least squares refinement are about 0.08 Å for individual iron and iron-sulfur bonds in both proteins (3, 7). Root mean square deviations from the mean values for chemically equivalent bonds in the cluster are slightly greater (12), but these are valid estimates only if the Fe&* clusters are tetrahedrally symmetric. Iron and sulfur positions in the two proteins appear to be sufficiently well defined by least squares refinement to reveal deviations of about 0.10 Å or more from tetrahedral symmetry, provided the differences in average bond length exceed 2.5 times the standard deviation of the mean value of the largest subset of equivalent bonds resulting from the distortion. Putative geometric asymmetry of ferredoxin Fe&*S4 clusters is also indicated by the fact that its two crystallographically independent clusters show similar distortions that are related by the intramolecular pseudo 2-fold axis.

Circular Dichroism Spectroscopy – Chromatium HiPIP was equilibrated with 50 mM Tris/HCl, pH 7.8, by passage through Sephadex G-20, and diluted to a concentration of 1.5 × 10⁻⁶ M with the same buffer. This sample was diluted 40-fold into a thermostatted, 1-cm cylindrical quartz cell (Precision Cells, Inc.) at 4°C containing either 0.98 ml of 50 mM Tris/HCl, pH 7.80 (4°), or 75 μl of 0.5 M Tris/HCl, pH 9.85 (4°), and 0.9 ml of dimethyl sulfoxide (Fisher 9-136, spectranalyzed) after first recording baseline rotations of these solutions. Spectra were recorded using a Cary 16 spectropolarimeter fitted with a circular dichroism accessory. To minimize fluctuations in the base-line resulting from movement of the sample elevator, samples were introduced to the cell directly through an opening in the side of the instrument. Ambient conditions in the instrument were kept free of oxygen by maintaining a 50 p.s.i. flow of nitrogen which had passed through columns of potassium hydroxide pellets and activated Chemalog R3-11 catalyst (Chemical Dynamics Corp., South Plainfield, N. J.) at 100°C. Spectra were digitized manually at intervals of 5 nm and corrected for base-line rotation.

RESULTS AND DISCUSSION

Equivalent orientations for a moiety such as FeS4*S4 atoms are determined by point group symmetry. Approximate tetrahedral symmetry for this group would evidently permit 12 nearly equivalent ways to superimpose the HiPIP cluster onto each ferredoxin cluster. Two questions follow from this consideration: (a) Is the true symmetry of the FeS4*S4 moiety itself actually less than tetrahedral? (b) If so, do the proteins make recognizable use of this reduced symmetry to bind the cluster in a unique way? Active site comparisons make sense if and only if both questions can be answered affirmatively. I have become convinced from studying analog FeS4*S4 cluster geometries (9, 16) that the symmetry of analog clusters in the paired-spin oxidation state is low enough in crystals that they have a unique orientation, i.e. a symmetry number of one, even without surrounding polypeptide. Moreover, this unique orientation can be recognized from qualitative features in the two protein crystal structures without referring to individual iron-sulfur bond lengths.

 Analog FeS4*S4 Cluster Atoms Are Distinguishable – Several authors have noted that the apparent point group symmetry of paired-spin FeS4*S4 clusters is lower than that of the tetrahedral group Td (4 3,) expected for such a compound (2, 9, 16). Analog crystal structures are sufficiently precise to reveal perturbations from this ideal symmetry. The most obvious deviation is tetragonal and flattens the FeS4* cube along a direction roughly parallel to four Fe—S* bonds, which are therefore shorter than the remaining eight Fe—S* bonds (Table I, Columns 1 and 2). A trigonal distortion involving primarily the terminal S8 sulfur ligands, and which has not been noted previously, is superimposed on this tetragonal distortion of the cube itself. Specifically, the tetrahedron formed by these four ligands has a unique base and apex: the three S8-S8 distances in the base average approximately 6.3 Å, those to the apex average approximately 6.5 Å (Table II, Columns 1 and 2). Taken together, the tetragonal distortion of the FeS4* core and the trigonal distortion of the S8 ligands provide basis vectors for a unique, internal coordinate system for the cluster. Thus, the tetragonal distortion preserves only one of the three 4-fold rotation axes, and the trigonal distortion preserves only one of the four 3-fold rotation axes characteristic of tetrahedral symmetry. The vector product of unit vectors along these two directions defines the z axis of this coordinate system. Unique 3 and 4 axes are, strictly speaking, mutually exclusive of one another and eliminate all 2-fold rotation axes. Thus the only symmetry operation relating all 12 FeS4*S4 atoms is the mirror plane normal to the molecular z axis as defined above. The molecular point group of the 12 cluster atoms is therefore C4 (ii).
Stereochemistry of Iron-Sulfur Protein Active Sites

### TABLE I

Tetragonal distortion of Fe₅S₅* atoms in paired-spin state clusters

<table>
<thead>
<tr>
<th>Analogue clusters</th>
<th>Protein-bound clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>R=CH₃*</td>
<td>HP₆s*</td>
</tr>
<tr>
<td>R=CH₂=C₃H₅*</td>
<td></td>
</tr>
<tr>
<td>A. Axial Fe—O* bond lengths (Å) (average of 4)</td>
<td>2.267 2.229</td>
</tr>
<tr>
<td>Off-axial Fe—S* bond lengths (Å) (average of 8)</td>
<td>2.296 2.310</td>
</tr>
<tr>
<td>σₐ = 0.02</td>
<td>σₐ = 0.04</td>
</tr>
<tr>
<td>B. Axial Fe—Fe bond lengths (Å) (average of 4)</td>
<td>2.731 2.732</td>
</tr>
<tr>
<td>Off-axial Fe—Fe bond lengths (Å) (average of 8)</td>
<td>2.745 2.770</td>
</tr>
<tr>
<td>σₐ = 0.02</td>
<td>σₐ = 0.01</td>
</tr>
</tbody>
</table>

* Ref. 16.

### TABLE II

Trigonal distortion of Sy ligands in paired spin state clusters

<table>
<thead>
<tr>
<th>Analogue clusters</th>
<th>Protein-bound clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>R=CH₃*</td>
<td>HP₆s*</td>
</tr>
<tr>
<td>R=CH₂=C₃H₅*</td>
<td></td>
</tr>
<tr>
<td>Basal Sy—Sy distances (Å) (average of 3)</td>
<td>6.27 6.32 (6.22)' (6.26)</td>
</tr>
<tr>
<td>Apical Sy—Sy distances (Å) (average of 3)</td>
<td>6.59 6.51 (6.40)</td>
</tr>
</tbody>
</table>

* Ref. 16.

### Theoretical Reasons for Loss of Symmetry

Theoretical reasons for loss of symmetry have been a subject of speculation (4, 17) and could influence the likelihood that unique 3 and/or 4 axes would persist inside the asymmetric environments of the polypeptide cluster-binding cavities. A plausible reason is that paired-spin cluster geometries of higher symmetry are unstable with respect to molecular vibrations according to the Jahn-Teller theorem (18). This explanation was first proposed to account for the apparent increase in cluster symmetry observed on oxidation of HP₆s to HP₆ (4) and it is supported by recent theoretical models for the paired-spin cluster which indicate electronic degeneracy of the highest occupied molecular orbitals in tetrahedral symmetry (17). Since reduced analog cluster symmetry may not result entirely from crystal-packing forces, we might hope to identify similar features in the protein-bound clusters. That is to say, a cluster with effective C₃ point group symmetry, in contrast to one with Tₐ symmetry, can in principle be recognized in a unique way by a polypeptide chelate in which the cysteinyl ligands are distinguished by their position in the primary sequence.

**Orientations of Protein-Bound Clusters**

Orientations of Protein-Bound Clusters ~ It is proper to use symmetry arguments in discussing the HiPIP and ferredoxin active sites, even though protein-bound Fe₅S₅*Sy₄ clusters cannot have strict symmetry elements. One can specify local symmetry by considering only the atomic positions of chemically equivalent atoms and ignoring the remainder of the polypeptide chain. Alternately, one can use the concept of effective symmetry to describe the highest possible symmetry that is consistent with other experimental observations obtained, for example, from optical or resonance spectra (19), and from analysis of structural features of the polypeptide chain. Unfortunately, neither local nor effective symmetries of protein-bound iron-sulfur complexes can be determined with certainty from available experimental data. Protein crystal structure data must be interpreted cautiously. A rather well known illustration is provided by the example of Fe—Sy distances in oxidized rubredoxin. These distances now appear to be equal within experimental error (20); previously, they appeared significantly unequal (21). The local symmetry of the Fe-Sy complex in this protein may therefore be quite high. Effective symmetry in protein-bound clusters will depend on electrostatic and dynamic, i.e. vibrational, effects introduced by the polypeptide chain, as well as on the local symmetry of the clusters themselves. In view of these possibilities, it is obviously of interest that spectroscopic analyses of oxidized rubredoxin (19) have revealed both trigonal symmetry in its vibrational spectra and tetragonal symmetry in its electronic spectra. An obvious suggestion, based on foregoing arguments, is that the only effective symmetry element in this iron-sulfur complex is also a mirror plane.

What, then, are the possible symmetry properties of the HiPIP and ferredoxin Fe₅S₅*Sy₄ clusters? Preliminary resonance-enhanced raman spectra of both proteins are qualitatively inconsistent with full tetrahedral symmetry (22). Data in Tables I and II illustrate the extent to which protein-bound cluster geometries at current resolution limits exhibit tetragonal and trigonal distortions similar to those observed in analog clusters.

L. H. Jensen, personal communication.
Each protein-bound cluster contains a set of four parallel Fe—S° bonds whose average length is approximately 0.1 Å less than the average of the remaining eight bonds (Table IA). Assuming that the eight long bonds derive from the same population and hence should be of equal length, the average short bond is shorter than the average long bond by 5σm, 1.75σm, and 2.75σm in HPred, Fdβ1, and Fdα1. The hypothesis that each protein-bound cluster therefore contains a 4 axis is supported by the following observations. Iron-iron distances in the protein-bound clusters are also significantly asymmetric. In ferredoxin the two iron-iron distances perpendicular to the 4 axis are longer than the other four by 11σm (cluster I) and 4σm (cluster II). The asymmetry in HiPIP is 4.5σm, and the tetragonal arrangement of the 4 iron atoms is not parallel to the 4 axis of the Fe,S° core as a whole. Iron atoms in analog clusters show less pronounced tetragonal distortions (Table IB). In one analog (R=CH2Phe) this distortion is parallel to the 4 axis defined by Fe—S° bonds; in the other (R=Phe) it is not. Finally, 4 axes in ferredoxin are parallel to one another, and therefore affect iron and sulfur atoms which are related by the intramolecular pseudo 2-fold axis.

Curiously, the subset of four Fe—Fe distances in HiPIP is long, rather than short (Table IB). There is therefore some evidence that although symmetry properties are maintained in the HiPIP cluster, it differs from all others in the distribution of valence electrons between bonding and antibonding iron-iron molecular orbitals.

There is a clear choice in HiPIP for a unique 3 axis. Average basal (6.24 Å) and apical (6.55 Å) Sy—Sy distances in HPred are in surprisingly good agreement with those in the two analog structures (Table II). The independently determined HPox structure indicates the same choice; basal and apical Sy—Sy distances average 6.18 Å and 6.45 Å. Moreover, if HPox and HPred data are averaged, and σm is defined as in Table I, average basal and apical distances differ by 5.8σm when each remaining Sy atom is chosen for the apex. However, both ferredoxin cluster geometries are consistent with more than one choice for the apical Sy atom (Table II, Columns 4 and 5). Thus, these data do not help to define a unique set of equivalent Sy atoms for the purpose of superimposing the protein active sites.

Remarkably, however, an approximate 3-fold symmetry element in the coordination geometry of cysteinyl ligands in HiPIP coincides with the unique 3 axis of the Sy atoms positions. Three cysteine residues (Cys 46, 63, 77), precisely those whose Sy atoms form the base assumed in Table II, are followed by hairpin turn conformations which orient backbone NH groups so as to donate NH—Sy bonds to the Sy atoms (4). The apical Sy atom (Cys 43) is removed from any such hydrogen bond donors. Similarly in ferredoxin (13), three of the cysteinyl ligands to each cluster find hydrogen bond donors (Cys 8, 11, 45 in Fdα1; Cys 18, 35, 38, in Fdα1) and one does not (Cys 14 in Fdα1; Cys 41 in Fdα1).

Patterns of NH—Sy hydrogen bonding in the active sites of both proteins are therefore structurally complementary to a cluster having reduced symmetry as observed in the more accurately known analog structures, and average dimensions of the protein-bound clusters conform quite well to these features. Further crystallographic analysis is unlikely to change the location of NH donors in either protein, so it is reasonable to assess the consequences of superimposing the two active sites as follows: define equivalent Sy atoms in HiPIP and ferredoxin by first identifying the two apical Sy atoms, those which lack NH—S hydrogen bonds, and then rotating about the unique 3 axes to align the 4 axes apparent in their Fe,S° cores.

Fig. 1 illustrates the relationships which have been derived above and identifies equivalent Sy atoms used to superimpose the HiPIP and ferredoxin molecules. Each cluster atom can be uniquely specified and has therefore been numbered according to a convention used previously to describe the HiPIP cluster (Ref. 4, Fig. 1). As an example, the cluster atoms Sy1, Fe1, Sy2, Fe2, S°1, and S°2 all lie in the unique mirror plane containing the effective 4 and 3 axes.

Analogies between HiPIP and Ferredoxin Active Sites— Analogies in the disposition of both main chain and side chain elements in the two proteins reinforce the foregoing assumptions regarding the effective cluster symmetry. To carry out the following analysis in a common coordinate system for the two protein models, equivalent cluster atoms (4Fe, 4S°, 4Sy) were first superimposed by a least squares procedure. The oriented molecules were then examined using the University of North Carolina Molecular Graphics Laboratory.

Only one of the two possible ferredoxin active sites was examined. However, there can be little doubt that the two active sites in ferredoxin are similar enough that they can be considered to be equivalent for the present purpose. Rossmann and Argos (23) have shown recently that polypeptide chain segments binding the two clusters are related by a surprisingly strict intramolecular pseudo 2-fold rotation axis. The HiPIP cluster was superimposed onto the ferredoxin cluster bonded to Cys 18, Cys 35, Cys 38, and Cys 41 (cluster II) because this cluster binding segment is composed of a single, continuous polypeptide that is uninterrupted either by breaks in the chain or by cysteine residues bonded to cluster I.

The cluster binding segment in HiPIP is constructed from antiparallel β structure and hairpin turns (Ref. 4, Fig. 3). A remarkably similar theme appears in the framework of the ferredoxin active site cavity. The comparison is illustrated in Figs. 2 and 3.

Several common features underly the two arrangements. First, basal sulfur atoms Sy2, Sy3, and Sy1 (Fig. 2B) arise from consecutive cysteine residues in both sequences (Fig. 2A). Moreover, both polypeptides follow a counterclockwise path when viewed from Sy1, as in Fig. 2B. Several different examples are known of consistent handedness in tertiary folding of polypeptides (24, 25); Fe,S° cluster binding may represent another example. Second, the stippled loops are approximately the same length in both proteins. (Although they are exactly the same length (18 residues) in Chromatium HiPIP and Pseudomonas aerogenes ferredoxin, these loops are of variable length in homologous proteins of both types (26, 27).) Third, the main chain conformations of the two loop segments, illustrated in Fig. 3, are quite similar in the two proteins. Both assume twisted, antiparallel β sheet conformations, despite the fact that they show no detectable amino acid sequence homology.

Parenthetically, it should be noted that the amino acid sequence of this loop in ferredoxin represents a cyclic permutation of what is generally held to be a duplicated gene (27). That is, it begins with the COOH terminus of the first copy and ends with the NH terminus of the second copy of the repeated sequence (Fig. 2A). This observation was, of course, implicit in the initial discovery (5) that neither Fe,S° cluster in ferredoxin was bonded entirely to four consecutive, closely
FIG. 2. Relative positions of equivalent cysteine residues in the HiPIP (Hp) and ferredoxin (Fd) sequences. A, cysteine positions in the primary structures of HiPIP and ferredoxin represented in the manner of Fig. 7 of Ref. 6. B, schematic showing the course of the polypeptide chains in the cluster binding segments. The stippled portion of chain appears on the lefthand side of Figs. 3 and 4. The solid arrow denotes the course of the chain near the apical cysteinyl residue whose Sy atom (Sy) lies on the effective 3 axis. The apparent 4 axis is indicated as in Fig. 1. Solid circles, iron atoms; open circles, inorganic sulfur atoms; hatched circles, “basal” sulfur atoms; dotted circles, “apical” sulfur atoms.

FIG. 3. Analogous main chain loops in HiPIP and in ferredoxin. Fe₄S₄ clusters are viewed from an identical reference point. Cysteine side chains and main chain elements within the analogous loops indicated in Fig. 2 are drawn with heavy bonds. NH–Sy hydrogen bonds to the basal Sy atoms are shown in heavy dotted lines. The viewing direction is approximately parallel to the effective 4 axis.

spaced cysteine residues in the primary sequence. From an evolutionary standpoint, this permutation of structurally necessary information away from the primary sequence is very intriguing. Have functional ferredoxins always required either an inter- or intramolecular dimer? Or has some genetic event changed the points of initiation and termination from those which existed in the ancestral, monomeric ferredoxin gene? Such questions are brought into focus by analogies illustrated in Fig. 3; answers will require additional comparative and functional studies.
There are also important differences between the two proteins. First, the apical $S_y$ atom, i.e. that which is not involved in NH—$S_y$ hydrogen bonding, arises from the first cysteiny1 residue (Cys 43) in the HiPIP sequence, and from the last (Cys 41) in the ferredoxin sequence. Both apical cysteine residues are connected via dipeptides to nearby cysteines, but the chains run in opposite directions (apical to basal in HiPIP, basal to apical in ferredoxin; see the heavy arrows in Fig. 2, A and B). Second, there is a shift in the relative orientations of both long and short loops relative to the Fe$^{3+}$ cluster. In ferredoxin both main chain elements are rotated clockwise by about 120º from their positions in HiPIP (compare Fig. 2B and Fig. 4). As a result, although equivalent atoms of each cluster superimpose upon one another with a root mean square deviation of 0.4 Å, main chain carbon atoms differ by a root mean square deviation of about 11 Å. This difference is correlated with a similar shift in amino acid side chain packing near the clusters, which will be described in the following paragraph.

Considerable discussion has centered on the fact that tyrosine residues pack close to the Fe$^{3+}$ clusters in the two proteins, and that these amino acids are conserved or of limited variability in homologous HiPIP and ferredoxin sequences (4, 6, 26-29). No assumptions regarding the orientation of these residues with respect to the Fe$^{3+}$ clusters were made in order to superimpose the two active sites. It is therefore probably significant that these side chains turn out to be associated with equivalent $S^*$ atoms ($S_y^*$, Fig. 4). They are not, however, associated with equivalent Fe$^{3+}$ faces, in contrast with a previous suggestion (4). Tyr 2 in ferredoxin occupies a position that is about 120º in a clockwise sense away from the position occupied by Tyr 19 in HiPIP, and is therefore shifted in the same way as are the main chain segments (Fig. 2B). The two tyrosine hydroxyl groups point in roughly opposite directions. Although not shown in Fig. 4, other side chains which tend to be conserved in homologous sequences of both types also pack close to equivalent $S^*$ atoms. Notably, Leu 65 (Hp) and Ile 36 (Fd) are in close proximity to $S_y^*$, and Met 49 (Hp) and Ile 30 (Fd) are in close proximity to $S^*$.

An important question should be raised at this point. How might the polypeptides select and stabilize a unique cluster orientation? Part of the answer may be suggested by the correlation between local 3 axes in the $S_y$ atom positions, and those cysteinyl residues which find nearby NH donors. What then determines the orientation of the local 4 axis? The complete pattern of six NH—$S$ hydrogen bonds in HiPIP conforms to the single mirror plane of the cluster. They occur in pairs, one of which is donated from each side of the mirror plane (Fig. 4, Hp). Moreover, the polypeptide framework in HiPIP is itself suggestive of reflection symmetry: antiparallel $\beta$ structures delimit opposite walls of the cluster binding cavity (4). In ferredoxin, NH—a $S$ hydrogen bonds do not conform to an approximate mirror plane, and cannot be responsible for selecting an orientation for the 4 axis. It also may be significant that ferredoxin Fe$^{3+}$,$S^*$,$S_y$ atomic positions themselves are more ambiguous with respect to the idealized, effective symmetry elements (Tables I and II). In short, it appears that the HiPIP polypeptide allows an environment complementary to the cluster's unique mirror plane, insofar as it is possible using only L-amino acids, and that no simple rationalization can be given at this time for the observed cluster orientation in ferredoxin.

**Broken Symmetry and Catalysis—**One interpretation of these comparative observations stands out above others in its simplicity, and in its novel implications for understanding how the two proteins catalyze different electron transfer reactions.

In order to place this interpretation into proper perspective it is useful first to review differences between the two proteins.
as they are currently understood. Considerable emphasis has been placed on understanding how the proteins regulate the midpoint potentials of the cluster. One mechanism, increased NH—S hydrogen bonding to reduced forms of the cluster (4, 12, 13), appears to be the most important factor in this phenomenon. The argument stems from three observations. First, NH—S hydrogen bonds can be identified in both proteins and there are more possible hydrogen bonds in ferredoxin than there are in HiPIP. Second, hydrogen bonds observed in oxidized HiPIP shorten by about 0.2 Å in reduced HiPIP (4). Third, approximate midpoint potentials are known for C+ + e− → C and C + e− → C reactions as observed in both proteins and in a large family of analog clusters (30). Potentials for the two successive reductions of a C+ cluster are separated by approximately the same energy in HiPIP as in the analog clusters (31). However, the relative midpoint potentials for equivalent reactions differ appreciably between proteins themselves, and between protein-bound and analog clusters. As shown in Fig. 5, the most consistent correlation to be found between structural features and observed potentials for the reaction C + e− → C involves the number of possible NH donors available to each cluster (12). This correlation is quite good; values estimated for the energy obtained by forming or strengthening an NH—S hydrogen bond range from 0.072 V/NH to 0.080 V/NH, suggesting a reasonable value slightly less than 2 kcal/mol of NH.

An important observation is that the potential measured for C + e− → C in HiPIP, measured under altered solvent conditions (31), also fits this correlation. It is unlikely that the C− state is more stable in the altered solvent conditions, one role of the native conformation being to stabilize reduced forms of the cluster through NH—S hydrogen bonding (Fig. 5). Since analog and ferredoxin paired-spin state clusters can undergo rapid reduction at midpoint potentials either lower or higher than that for HiPIP this observation is prima facie evidence for catalysis in native HiPIP. In other words, the paired-spin cluster in native HiPIP cannot be reduced to the C− state because there is a kinetic barrier, and not because its midpoint potential is too low. Moreover, it is hard to see how differences in the number or strength of NH—S hydrogen bonds in the two proteins could be involved in such a barrier. The HiPIP polypeptide must destabilize the transition state for super reduction by means of other, as yet unidentified mechanisms.

Comparison of active site stereochemistries in the two proteins reveals previously unsuspected differences which can help to explain their different catalytic properties. The Fe6S43+Sn cluster evidently shares with numerous other biological substrates the stereochemical property known as prochirality (32). One way to appreciate this is to recall that a unique mirror plane divides the cluster into two nonsuperimposable halves which can be distinguished by a chiral reagent such as a polypeptide. Indeed, I have utilized this fact in superimposing the two protein active sites. An additional consequence of prochirality is that once bound to the protein, the cluster will experience an asymmetric environment and hence may become measurably chiral. Similarities in the main chain geometry illustrated in Fig. 2B suggest that the hand of this influence is, in at least one respect, the same in both proteins. The polypeptide chain direction and hence the polarity of NH—Sy hydrogen bonds from residues N + 2 convert the three basal Sy atoms into a propeller which has the same sense in both proteins (Fig. 2B). A natural stereochemical priority for Sy atoms (1 → 2 → 3 → 4) is provided by the numbering system of the cysteine sulfur atoms in Fig. 1. In accordance with the well known chirality rule (33) the asymmetry of the main chain around the cluster can be assigned the symbol S in both cases.

In contrast, perturbations of Fe6S4+ core atoms introduced by the proximity of tyrosine residues are clearly of the opposite handedness in the two proteins. Note in Fig. 4 that the shaded Fe6S4+ faces, representing interaction with tyrosine side chains, are reflected into one another by the effective mirror plane and are therefore diastereotopic faces. Disregarding the asymmetry of the dual-sulfur itself as well as its position in the polypeptide chain, the asymmetry of the tyrosine cluster interaction may be specified S or R as outlined in Ref. 32 for trigonal faces. View the inorganic sulfur atom Sn, taken as the chiral center, from within the cluster and assign highest priority to the iron atom closest to the tyrosine Cα atom (Feα in HiPIP, Feα in ferredoxin). The resulting sequences are Feα > Feβ > Feγ > Feδ > phantom ligand on Sn, i.e. S for HiPIP; and Feγ > Feδ > Feβ > Feα > phantom ligand on Sn, i.e. R for ferredoxin. Association of corresponding sulfur atoms, Sn, with tyrosine side chains from opposite sides of the effective mirror plane, in combination with the asymmetry of the main chain (see above), creates a diastereomeric pair, S-S and S-R, in the Fe6S4+ cluster of HiPIP on the one hand and ferredoxin on the other. Factorization of the asymmetry induced by qualitative features of the two cluster-binding segments bears an obvious and striking similarity to the manner by which the point group symmetry of analog clusters is reduced from T3m to C2m by combining the effects of trigonal and tetragonal distortions of different sets of equivalent atoms.

Casting two different protein active sites as diastereomers is, to be sure, a simplification of the true relationships which distinguish the two proteins because it disregards sequence and conformational differences between them. The following considerations suggest the degree to which such simplification may be justifiable.

1. Steric and polar influences from the protein should affect...
sulfur atoms most strongly. These atoms have larger van der Waals radii (1.85 Å) than do the iron atoms (~0.70 Å) (34). Thus, these atoms account for most of the surface area approached by protein groups, a fact that has been obscured by a tendency to represent the cluster as a cube. Moreover, mutual repulsion between sulfur electrons also means that these atoms are considerably more polarizable than are the iron atoms at the core of the cluster (35).

2. Groups in the cluster-binding cavity which will exert the greatest influence on the cluster are probably the fixed dipole moments of backbone NH groups involved in NH–S hydrogen bonding and induced dipole moments in the aromatic rings. These are just those elements which occur to be oriented so as to induce chirality in the cluster. Features which have been ignored in advancing the argument would be expected to exert much smaller influences, or to reinforce the primary ones. In addition to Tyr 19, HiPIP has three other aromatic rings which are in the cluster (Fig. 4 of Ref. 4). The indole ring of Trp 76 approaches S* in much the same way as does Tyr 19 to S*. S* is not in the mirror plane; it is on the same side of the plane as is Tyr 19. The effects of Tyr 19 and Trp 76 are therefore probably synergistic rather than antagonistic with respect to the cluster’s chirality. The edges of Phe 66 and Phe 48 approach S* and S* from directions almost parallel to the mirror plane. Ferredoxin has no aromatic groups other than its two tyrosines, and as noted above is similar to HiPIP in the location of its nonpolar side chains. Two NH–S hydrogen bonds to the ferredoxin cluster involve S* (Fig. 4). Curiously, these hydrogen bonds occupy a position that is enantiomeric (in the sense used to relate the respective tyrosine positions) to the position of Phe 66 in HiPIP.

3. Several lines of evidence indicate that electronic perturbation of the tyrosine side chain by the cluster is intense in both proteins, suggesting a strong tyrosine-cluster interaction. Ring carbon 13C nuclear magnetic resonance of tyrosines in Clostridium acidi urici ferredoxin are shifted downfield relative to their positions in free tyrosine (28). No similar study has been published for HiPIP, but there is crystallographic evidence from comparing oxidized and reduced HiPIP that an unusual NH–O hydrogen bond to the tyrosine hydroxyl group becomes stronger in the reduced protein. This observation suggests that negative charge is delocalized onto the hydroxyl group in the reduced state and has been interpreted to mean that a charge-dipole interaction occurs between tyrosine and cluster (4).

4. There is a 25% improvement in the root mean square deviation of the 12 cluster atoms when the reduced HiPIP cluster is reflected through the effective mirror plane before it is superimposed by least squares on an oxidized ferredoxin cluster. Enantiomeric influences in the two proteins, i.e. the tyrosine-cluster interactions, may therefore have measurable effects on cluster geometry, suggesting a reciprocal electronic perturbation of the clusters by the tyrosine side chains.

5. The role ascribed to tyrosine in this scheme, namely to impose handedness on Fe4S* orbitals, can be fulfilled by any of the amino acids (Phe, His, Arg) which have observed to replace tyrosine in homologous ferredoxin sequences (27). Enantiomeric influences in the two proteins, i.e. the tyrosine-cluster interactions, may therefore have measurable effects on cluster geometry, suggesting a reciprocal electronic perturbation of the clusters by the tyrosine side chains.

This conclusion is supported by available experimental data. Optical activity is a directly accessible manifestation of chirality by an asymmetric molecule. It is therefore of interest to compare the circular dichroism spectra of reduced HiPIP and oxidized ferredoxin as shown in Fig. 6. The native HiPIP spectrum, recorded at 4°C in 50 mM Tris buffer, pH 7.8, is in good agreement at most wavelengths with published spectra (36), but should not be interpreted below about 320 nm because of high absorption by the protein. The long wavelength part of this spectrum is also qualitatively similar to the same region in the ferredoxin spectrum (redrawn from Ref. 37). Broad bands with positive ellipticity at about 600 nm and at 458 nm in HiPIP correspond to similar bands at 576 nm and 455 nm in ferredoxin. The HiPIP and ferredoxin spectra differ dramatically in the short wavelength region indicated by cross-hatching in Fig. 6. Between 320 nm and 450 nm the two curves have opposite curvature, reduced HiPIP having two bands with negative ellipticities at 348 nm and at 384 nm and oxidized ferredoxin having two bands with positive ellipticities at 325 nm and at 415 nm.

Analog clusters are probably either prochiral, as I have suggested, or achiral. In neither case would they be expected to show appreciable optical rotation. Yet the optically active bands shown in Fig. 6 almost certainly arise from electronic transitions in the Fe4S*Sy4 clusters. The optical absorption corresponding to these bands has been assigned on the basis of theoretical calculations (17) to S* to Fe charge-transfer bands. As noted above, the highest energy S* electrons are highly polarizable. These charge-transfer bands are therefore likely to develop induced rotational strength when perturbed by the polypeptide environment. Bands in the cross-hatched

![Figure 6. Circular dichroism spectra of native reduced HiPIP (---) and super-reducible HiPIP (---) at 4°C, and of oxidized ferredoxin (---) at 5°C. Molar ellipticity, [ε], is defined by the expression: [ε] = 1000 [θ]/(MlC), where θ is the measured ellipticity in degrees, M is the molecular weight, l is the path length in centimeters, and C is the concentration in grams/ml. All ellipticities are expressed per dmol of Fe4S*Sy* cluster. The ferredoxin spectrum was redrawn from Ref. 37. Cross-hatched regions show transitions with opposite rotation in the two proteins. Me2SO, dimethylsulfoxide.](http://www.jbc.org/)

Fig. 6. Circular dichroism spectra of native reduced HiPIP (---) and super-reducible HiPIP (---) at 4°C, and of oxidized ferredoxin (---) at 5°C. Molar ellipticity, [ε], is defined by the expression: [ε] = 1000 [θ]/(MlC), where θ is the measured ellipticity in degrees, M is the molecular weight, l is the path length in centimeters, and C is the concentration in grams/ml. All ellipticities are expressed per dmol of Fe4S*Sy* cluster. The ferredoxin spectrum was redrawn from Ref. 37. Cross-hatched regions show transitions with opposite rotation in the two proteins. Me2SO, dimethylsulfoxide.)
regions of Fig. 6 occur at similar wavelengths in the two proteins but have opposite signs, suggesting that these rotations arise from enantiomeric interactions, for example, those between tyrosine side chains and the cluster.

Diastereoisomerism can, in principal, differentiate the chemistry of the protein-bound clusters. Does it do so in a way which would help to explain their contrasting oxidation-reduction properties? On the basis of several intriguing correlations involving the generation and properties of super reducible HiPIP at pH > 10 in 85% dimethylsulfoxide, I believe the answer is yes. When HiPIP at pH 9.85 is transferred to 85% dimethylsulfoxide there is a general reduction in the ellipticities of all dichroic bands (Fig. 6), the most striking changes occurring in the two negative bands at 394 and at 348 nm, and in the positive band at 458 nm. Transition to super reducible HiPIP is therefore accompanied by the loss in rotational strength of certain optical absorption transitions lying between 280 nm and 500 nm.

Transition to super reducible HiPIP occurs in a solvent with greatly reduced water activity and at high pH. There is some uncertainty regarding the precise pH at which this was measured. Although the protein solution was buffered with 50 mM Tris buffer at pH 9.85 (44), control experiments suggest that the actual pH is 10.5. Transition to the super reducible state is very pH-dependent and did not occur when aqueous protein solutions buffered at pH 9.2 or 9.5 (44) were diluted to 85% dimethylsulfoxide, in quantitative agreement with a previous report (31), which required a Tris pH greater than 9.0 (20°) for the transition. This pH dependence suggests that the transition to super reducible HiPIP requires titration of a protein functional group whose pKₐ is about 10. One such group is the Tyr 19 hydroxyl group.

The environment of Tyr 19 in native reduced HiPIP is such that it would be strongly disrupted by titrating its hydroxyl group and perhaps by dehydration. Specifically, as described in Ref. 4, the hydroxyl group accepts an NH-O hydrogen bond from Nₓ and donates an OH-O hydrogen bond to a bound water molecule. Disruption of this hydrogen bonding network would obviously destabilize the proposed charge-dipole interaction between Tyr 19 and the anionic FeₓSₓ⁺⁺ cluster, and could therefore account for the loss of rotational strength observed in super reducible HiPIP. Two observations strengthen this interpretation. First, crystallographic evidence (4) indicates that the tyrosine-cluster distance increases in oxidized HiPIP, which also shows reduced rotational strength in the two negative bands (470 nm) and the positive band at 348 nm. Loss of rotational strength in the region of sulfur to iron charge transfer bands can therefore be demonstrated both for HiPIP and for related FeₓSₓ⁺⁺Syₓ clusters under nondenaturing conditions. The former example establishes a correlation between rotational strength and physical intimacy of the tyrosine cluster interaction; the latter establishes a correlation between rotational strength and catalytic behavior. An intriguing corollary possibility is that the altered active site configuration underlying the loss of rotational strength and also, presumably, transition to super reducible HiPIP may not involve extensive denaturation of the polypeptide chain.

On tyrosine residues in either protein provide a direct orbital pathway for electron transfer? The foregoing observations are consistent with such mechanisms (4) or with other sites of electron transfer. They suggest only how perturbations of cluster orbitals by protein groups might influence the relative reactivity of protein-bound FeₓSₓ⁺⁺Syₓ clusters toward oxidants and reductants. Diastereomeric elements of protein structure surrounding the HiPIP and ferredoxin clusters can be expected on well established grounds to elicit different physical and chemical properties from the same prosthetic group. In HiPIP, one consequence of this difference may be to permit an unusual charge-induced dipole interaction to occur between the negatively charged cluster and a tyrosine side chain. This interaction, in turn, could have three effects. First, it may prevent the irreversible degradation which normally ensues after oxidizing paired-spin analogs and ferredoxin FeₓSₓ⁺⁺Syₓ clusters to the C⁺ state (11). Second, as suggested above, it may increase the activation energy for reduction of the paired-spin state, effectively preventing the low potential reaction, C + e⁻ → C⁻. Finally, it may lower the activation energy for oxidation, and thereby facilitate the high potential reaction C⁺ + e⁻ → C (see also points 1 and 2 below). Quantum mechanical interactions between chiral wave functions and how they distinguish the chemical reactivity of diastereomers are complex and largely unexplored research areas (39). Until such details can be given, these conclusions will remain, to a large extent, descriptive.

This shortcoming is not surprising, since both the active site superposition, and the stereochemical comparisons have been simplified by symmetry principles. Although they are of great heuristic value, arguments based entirely on symmetry tend to obscure aspects of protein structure which must be ignored in order to apply them. The following observations cannot yet be incorporated into the present picture of iron-sulfur protein chemistry and function.

1. There would still be important differences in the orientations of Tyr 19 in HiPIP and Tyr 2, in ferredoxin, even if the former were reflected through the mirror plane in the cluster (4). Interactions between Tyr 2 and Sₓ⁺⁺ in ferredoxin are symmetrical with respect to Cₓ and Cₓ⁺⁺, suggesting orbital overlap between lowest unfilled tyrosine and highest filled cluster molecular orbitals. The interaction in HiPIP is asymmetric, involving only Cₓ, and suggesting the reversed interaction between highest filled tyrosine and lowest unfilled cluster molecular orbitals.

2. Complementary influences may affect the electronic distribution within the FeₓSₓ⁺⁺Syₓ clusters. Four of the six NH—S hydrogen bonds in HiPIP (to Sₓ⁺⁺, Sₓ⁺⁺, and Sₓ⁺⁺) are directed toward the opposite end of the cluster from Tyr 19, whereas the edges of Phe 48 and Phe 60 are directed toward Sₓ⁺⁺ and Sₓ⁺⁺ at the same end. The situation is inverted in ferredoxin, three of the four NH—S hydrogen bonds being directed toward Sₓ⁺⁺ close to Tyr 2 (Fig. 4). These influences might induce cluster dipole moments in opposite directions, pushing electrons away from Tyr 19 in HiPIP and pulling them toward Tyr 2 in ferredoxin.

3. Consistent with these possibilities is, of course, the
observation that hydrogen bonding to the tyrosine hydroxyl group appears to be important in HiPIP but not in ferredoxin (4).

4. Finally, in HiPIP the principle molecular dipole moment of Tyr 19, \( \text{Cy} \rightarrow \text{OH} \), is parallel to an iron-iron bond, \( \text{Fe} \rightarrow \text{Fe} \); the corresponding moment in Tyr 2 is parallel to a sulfur-sulfur distance, \( S_2^* \rightarrow S_3^* \), in ferredoxin (Fig. 4).

A coherent catalytic scheme will require incorporating these and other as yet unrelated observations. A common substrate orientation for HiPIP and ferredoxin, together with the underlying symmetry principles, can be useful in working out these details for iron-sulfur proteins and perhaps for other electron transport proteins.

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