JBIC (1996) 1:2–14

MINIREVIEW

**Jean-Marc Moulis · Valérie Davasse
Marie-Pierre Golinelli · Jacques Meyer
Isabelle Quinkal**

The coordination sphere of iron-sulfur clusters: lessons from site-directed mutagenesis experiments

Abstract Cysteine is the ubiquitous ligand of iron-sulfur clusters in proteins, although chemical models have indicated that functional groups other than thiolates can coordinate iron in iron-sulfur compounds. Only a small number of naturally occurring examples of hydroxyl, histidinyl or carboxyl coordination have been clearly established but many others are suspected. Quite a few site-directed mutagenesis experiments have been aimed at replacing the cysteine ligands of iron-sulfur centers by other amino acids in various systems. The available data set shows that substituting one ligand, even by another functional residue, is very often destabilizing enough to impair cluster assembly; in some cases, the apoprotein cannot even be detected. One for one replacements have been demonstrated, but they have been so far almost exclusively confined to clusters with no more than one or two iron atoms. In contrast, changes of the cluster nuclearity or recruitment of free cysteine residues seem preferred ways for proteins containing larger clusters to cope with removal of a ligand, rather than using coordinating amino acids bearing different chemical functions. Furthermore, the possibility of replacing cysteines by other residues as ligands in iron-sulfur proteins does not uniquely depend on the ability of the cluster to accept other kinds of coordination than cysteinate; other factors such as the local flexibility of the polypeptide chain, the accessibility of the solvent and the electronic distribution on the active centers may also play a prominent role.

Key words Site-directed mutagenesis · Iron-sulfur · Ligand exchange · Cluster conversion

J.-M. Moulis (✉) · V. Davasse · M.-P. Golinelli · J. Meyer
I. Quinkal
CEA, Département de Biologie Moléculaire et Structurale,
Laboratoire des Métalloprotéines, 17 rue des Martyrs,
F-38054 Grenoble Cedex 9, France
Tel.: +33 76885623; fax +33 76889808;
e-mail: moulis@ebron.ceng.cea.fr

Introduction

For the first two decades after their discovery (1960–1980), the inorganic cores of iron-sulfur (Fe-S) proteins were thought to be exclusively bound to thiolate groups provided by cysteine residues of their polypeptide chains. This view arose from the first X-ray crystallographic structures available [1–8], all from electron transfer proteins. In these cases no active chemistry involving the ligands of the Fe-S cores seemed needed since no substrate turnover was required. Moreover, differences in the redox reactivities of geometrically identical clusters in different proteins could be readily accounted for by other structural features [9].

However, it was soon demonstrated that Fe-S proteins participated in reactions involving the conversion of substrates like molecular nitrogen [10] or pyruvate [11]. The list has been increasing ever since, with the discovery of new functions for Fe-S proteins accelerating in recent years. In most cases, Fe-S clusters are associated with other types of cofactors, such as other metals (Ni, Mo, Fe, V etc.) or organic groups (FMN, FAD), and definitive proof for the direct participation of an Fe-S cluster in the binding and transformation of a substrate has long been lacking. Such evidence has now been obtained in at least one example (see below, "A conitase") and, in view of the increasing number of proteins that interact with substrates and contain Fe-S clusters only, other instances will probably soon be revealed.

Various aspects of the bioinorganic chemistry of Fe-S proteins are regularly reviewed, and recent comprehensive accounts are available [12, 13]. However, the rules governing ligand substitutions on Fe-S clusters in proteins have not received wide attention, in contrast to synthetic analogues [14]. It is the purpose of the present contribution to focus on recently reported site-directed mutagenesis (SDM) experiments carried out

on the ligands of Fe-S proteins[1]. Mixed metal clusters, such as the Mo-Fe cofactor of nitrogenase or the Ni-Fe cluster of some hydrogenases, are not included since the relevant reactivity heavily depends on the poorly understood coupling between iron and other transition metal ions. Therefore, only those clusters for which both the geometry and the electronic structure are relatively well known, such as the most common [1Fe], [2Fe-2S] and [4Fe-4S] clusters of rubredoxins and ferredoxins, are discussed. It is hoped that this compendium will provide guidelines for designing future SDM experiments on Fe-S proteins and for implementing means to interpret them.

Ligand exchange on synthetic compounds

The field of Fe-S protein chemistry soon benefited from the availability of synthetic analogues in which the cysteine ligands of the proteins were replaced by small organic thiols [15]. These compounds were obtained via self-assembly reactions in which ferric ions, sulfide (or elemental sulfur) and well-chosen thiolates spontaneously formed low-molecular-weight counterparts of the [2Fe-2S] or [4Fe-4S] active sites of proteins [14]. These initial successes led to the exploration of other potential terminal ligands in such structures. Among the numerous anions able to coordinate Fe-S cores, some provide functional groups which may be found in proteins; examples are phenolates, carboxylates and imidazolates [16–18]. Also, a significant Fe-O interaction between one of the metal sites and the *o*–hydroxybenzenethiol ligand of $[Fe_4S_4(SC_6H_4–o–OH)_4]^{2-}$ has been evidenced in the solid state [19]. The binding of esters of non-sulfur amino acids such as serine and tyrosine to Fe-S clusters has also been proposed [20].

A step forward in the synthesis of Fe_4S_4 analogues bearing non-sulfur ligands came from the design of appropriate tridentate thiols, leaving a single iron position available for ligand exchange. This differentiated position has been shown to bind acetate or hydroxide, as well as many other functional groups [21]. From all available studies on synthetic clusters, no major limitation in the type of ligand stabilizing Fe_2S_2 or Fe_4S_4 cores seems to exist for a skilled chemist. It is thus of the utmost interest to examine whether such diversity occurs in – or can be engineered into – proteins as a potential means of tuning the properties of Fe-S clusters.

The likeliness of ligand exchange is evidently related to the lability of the Fe-S bonds. Although this point has not been thoroughly investigated, data obtained on [4Fe-4S] ferredoxins show that the polypeptide can rapidly lose its cluster and reassemble it, if the required reactants are available [22, 23]. These results are comparable with those of similar studies carried out on synthetic analogues [24] and, together with other observations [25, 27], suggest that ligand exchange is indeed an easy process for, at least, simple Fe-S proteins. This property is one of the main factors explaining the widespread instability of these proteins and it sets this family apart from other metalloproteins organized around complex metal-containing cofactors, such as hemoproteins or diiron-oxo proteins, for which the exchange of *all* ligands is generally not an easy reaction. However, the lability of Fe-S clusters depends on both the type of cluster considered and on the polypeptide. For example, *Anabaena* [2Fe-2S] ferredoxin denaturation in guanidinium hydrochloride is a multistep process in which the destruction of the [2Fe-2S] cluster follows other structural changes [28]. Overall, neither what has been gathered over 20 years of chemical research on Fe-S clusters nor what is known of the chemical properties of such clusters in proteins suggests any reasons why non-cysteinyl ligands of Fe-S clusters should not occur in biological systems.

Naturally occurring non-cysteine ligands in proteins

Evidence from X-ray crystallography

Aconitase

A benchmark in the knowledge of the diversity of function and molecular mechanisms attainable by Fe-S clusters was set by the thorough studies carried out on aconitase. The enzyme from mammalian mitochondria belongs to the Krebs cycle and interconverts citrate and isocitrate. The involvement of an Fe-S cluster in the reaction has been demonstrated and characterized by a wealth of spectroscopic techniques including Mössbauer spectroscopy, EPR and ENDOR. One of the main outcomes of these studies has been the definition of the active [4Fe-4S] cluster environment in which oxygen atoms belonging to the solvent or to bound substrates interact strongly with one of the iron atoms [29, 30].

Further clues came from the resolution of the crystallographic structure of the enzyme. The active [4Fe-4S]-containing protein clearly showed coordination by three cysteine residues, but no ligand other than a solvent molecule within bonding distance of the fourth iron [31]. The latter iron is the one that is lost during aerobic purification of the protein to give the [3Fe-4S] form [32]. Growth of aconitase crystals in the presence of substrates or inhibitors [33–35] revealed that the same iron switched from tetrahedral to generally octahedral geometry, with the non-sulfur coordination provided by one monodentate carboxyl and one hydroxyl from the substrate/inhibitor, and one water molecule. Pentacoordinated iron occurred with trans-aconitate [34]. At the level of resolution achieved (around 2Å),

Whenever possible, reference will be made to recent reviews rather than to the original literature.

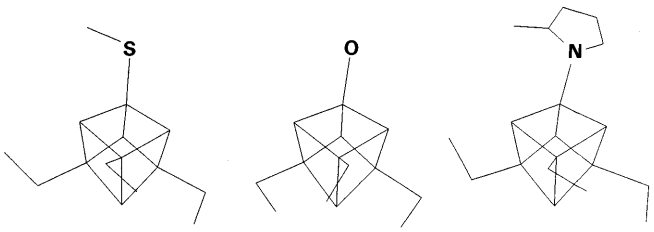

Fig. 1 Examples of non-sulfur coordination in [4Fe-4S] proteins. *Left* Coordination of the cluster by four cysteines, as in *Clostridium acidurici* ferredoxin [59]; *center* coordination by three cysteines and one solvent hydroxide in sulfate-complexed bovine mitochondrial aconitase [35]; *right* coordination by three cysteines and one histidine in *Desulfovibrio gigas* hydrogenase [36]. The ligand side chains are drawn up to the β-carbon only

tetrahedral coordination of the iron involving a single hydroxyl group (no substrate bound) does not lead to major distortion of the core structure (Fig. 1): only the Fe-hydroxyl distance is shorter by ca. 0.4 Å than the most common Fe-S_γ bond lengths [31–35]. Keeping $[Fe_4S_4(SR_4)]^{2-}$ clusters as references, hexacoordination of one iron in the presence of substrates or inhibitors induces a ca. 0.2 Å elongation along the diagonal of the Fe_4S_4 unit passing through the iron of the S_3FeO_3 site [33]. It was also found that the positions of the oxygen ligands vary considerably with the type of substrate/inhibitor used, in agreement with the changes in the iron coordination sphere proposed to occur during the catalytic cycle [34, 35].

Aconitase is thus the first definitive example of non-sulfur coordination for an Fe-S cluster obtained through the combination of detailed crystallographic studies and extensive spectroscopic investigations. In all other cases discussed below, current knowledge is mainly based on either one of these two approaches.

Hydrogenase

The recently elucidated structure of *Desulfovibrio gigas* hydrogenase has afforded, among other new insights into the bioinorganic chemistry of Fe-S and Ni proteins, evidence for the occurrence of one [4Fe-4S] cluster bound to the polypeptide chain by three cysteines and one histidine [36] (Fig. 1). Refinement of this structure at a resolution better than 2.85 Å is impatiently awaited so that the structural consequences of histidine coordination on the detailed geometry of [4Fe-4S] clusters might be more precisely evaluated.

Nitrogenase

New types of Fe-S clusters have also been disclosed by the structures of the proteins of the nitrogenase complex. In the large component of the system a cluster made up of two bridged [4Fe-4S] cores has been demonstrated, in addition to the Fe-Mo cofactor thought to be the site of substrate binding [37–40]. The so-called P-cluster pair is, therefore, the first documented example of an all-iron cluster expanding the classical nuclearity range beyond four metal ions. The environment of the eight irons consists almost exclusively of sulfur atoms, except for the side contributed by subunit β, which may provide a serine ligand to one of the irons [37–40]. This situation is reminiscent of the pentacoordinated iron atom shown in a synthetic analogue [19] (see above, "Ligand exchange on synthetic compounds"). However, an alternative interpretation of independently recorded X-ray diffraction data proposes that the same serine residue would hydrogen-bond to one of the inorganic sulfur atoms of the cluster (discussed in [41]).

Since the latter case is still a matter of controversy, the only clear examples of non-sulfur coordination obtained in X-ray diffraction studies involve [4Fe-4S] clusters (Fig. 1). However, this observation may be coincidental, as other Fe-S proteins are suspected to use non-cysteinyl ligands, according to data obtained with different methods.

Other evidence

Although often easier to implement, spectroscopic studies of Fe-S clusters do not generally give evidence for non-cysteinyl ligands as clear-cut as that from X-ray crystallography. However, the spectroscopic signatures of many systems suggest that cysteines may not be the exclusive ligands of Fe-S clusters in proteins, and these data will now be reviewed.

Rieske protein

The name 'Rieske protein' has been associated with an Fe-S protein of the [2Fe-2S] type found initially in complex III of mitochondrial respiratory chains, then in other membranous electron transfer chains of plants and bacteria [42]. More recently, components of some bacterial dioxygenase systems have been added to the group [43]. The most remarkable property of these redox proteins is their high reduction potential compared to conventional soluble [2Fe-2S] proteins with exclusively sulfur ligands.

In order to explain such discrepancies, spectroscopic techniques have been applied. It was soon demonstrated that the [2Fe-2S] cluster has apparently an usual geometry, but other differences with respect to $Fe_2S_2(SR)_4$–based proteins suggested that histidine ligation could be involved. Among relevant data, the unusual Mössbauer isomer shift of one iron of the cluster, EXAFS spectra, the low average value of the EPR g-tensor, nitrogen hyperfine couplings in the ENDOR and ESEEM spectra and the pattern of resonance Ra-

man bands all pointed to the more easily reducible iron being coordinated by two histidines [42, 43]. This would involve two short peptide segments of sequences CXH and CXXH (X = variable amino acid) as the coordinating units of the cluster, although two additional conserved cysteines are found nearby in the sequence (see below, "Cases in which no cluster is associated with the protein").

[4Fe-4S] ferredoxins

A very interesting development in the chemistry associated with Fe-S clusters in proteins was the discovery of [3Fe-4S]/[4Fe-4S] cluster interconversions [44]. It was then soon realized that some ferredoxins, e.g. *Desulfovibrio africanus* ferredoxin III, *Desulfovibrio vulgaris* Miyazaki and *Pyrococcus furiosus* ferredoxins (NCBI accession numbers 119957, 119924 and 517273, respectively), have a cluster-binding sequence of the CXXDXXC form with a remote cysteine available, instead of the similar segment with three cysteines: CXXCXXC (binding motif 1).

Since such proteins could be readily obtained in their [4Fe-4S] form, the question arose as to the nature of the ligands of the cluster. Evidence for the binding of exogenous ligands, such as thiols or cyanide, to these clusters has been obtained [45, 46]. Also, spectroscopic data suggest the binding of non-sulfur amino acids to the same clusters [47, 48]. However, definite evidence for aspartate coordination to one of the metal has come very recently from NMR studies of *Pyrococcus furiosus* ferredoxin [49]. At the reduced level, strongly hyperfine-shifted signals can be assigned to four spin systems, three corresponding to cysteines and one to a $-CH_2-CH$-fragment of the polypeptide chain, most probably belonging to the aspartate of the cluster binding motif. The data did not determine the coordination mode of the carboxylate, but suggested that the unique non-sulfur ligand may play a role in the electron transfer mechanism of the protein. Indeed, the regulation of the electron transfer rate of Fe-S clusters by the ionization or coordination state of amino acid groups (mainly carboxylates) is a matter of emerging interest [50].

Miscellaneous

Many other, often complex, proteins containing Fe-S centers are suspected of using non-sulfur amino acids as ligands. It is the case of enzymes whose spectroscopic and catalytic properties resemble those of a well-defined system, such as many dehydratases functionally related to aconitase [51]. In other instances, sequence comparisons associated with spectroscopic identification of the type(s) of Fe-S centers point to missing potential cysteine ligands: *Escherichia coli* succinate dehydrogenase subunit B displays an aspartate in place of a cysteine bound to the [2Fe-2S] cluster in other species [52]. The role of this residue has been probed by SDM [72].

Along similar lines, Fe-S clusters of elusive structure in a few proteins are suspected of including non-cysteine ligands. The supposedly substrate activating H-cluster of Fe-only hydrogenases [53] and the putative [6Fe-6S] prismane cluster containing protein from sulfate-reducing bacteria [54] belong to this group. Such predictions, when supported by SDM experiments, for instance, can be proven correct, as has been shown in the case of the Mo-Fe cofactor of nitrogenase [41].

Despite the increasing number of cases in which the involvement of non-cysteine ligands has been confirmed, the convincing determination of the nature of the ligands of Fe-S clusters requires a wealth of data obtained through a variety of methods. In fact, ultimate proof is seldom obtained by techniques other than the genuinely structural ones (X-ray crystallography and, in only one example so far, NMR). However, since these methods cannot be conveniently applied to all proteins, alternatives have to be sought, and SDM experiments are among the most efficient means of elucidating the coordination chemistry and reaction mechanism of Fe-S proteins. It is the purpose of the next section to summarize the currently available information on these points.

Information obtained from SDM experiments

In many cases, heterologous expression has been a prerequisite for the development of SDM of Fe-S proteins. Due in part to the instability of these proteins, this preliminary step is not always straightforward, and widely variable yields of recombinant Fe-S proteins have been observed. Many mutations have more drastic effects than mere quantitative change in the properties of the target protein: they can prevent the assembly of the Fe-S cluster or even impair the detection of the polypeptide. Such a classification can be applied to all published data available on Fe-S proteins modified on one or several of their ligands and will be used below.

Cases in which no peptide is detected or no multisubunit complex assembly occurs

In some cases, the introduction of an alien amino acid in place of a ligand of the Fe-S cluster cancels the possibility of expressing the mutated gene or impairs the correct assembly of the functional multisubunit complex. The transcription-translation series of events is generally not responsible for this situation, since a single, most often internal, mutation is unlikely to perturb the expression of a gene which is otherwise (with the native form for instance) well expressed. Rather, the changes involve post-translational events and are re-

lated to the folding of the protein. Indeed, the presence and correct insertion of an Fe-S cluster is often a determining factor in the folding and stability of the protein. When these properties are strongly impaired by the replacement of a ligand of the cluster(s), the expression host can efficiently degrade the nascent polypeptide, which is not only inactive but cannot even be detected.

A significant example is provided by the substitution of cysteine 565 by histidine and aspartate in the photosystem I protein PsaB of the cyanobacterium *Synechocystis* PCC6803 reaction center [55]. This single mutation in one gene of the complex induces the almost complete disappearance of all four polypeptides (PsaABCD) constituting photosystem I and the loss of phototrophic growth. The role of this cysteine as a ligand of the [4Fe-4S] cluster F_x was thus inferred and sustained by the results obtained with the complementary *Synechocystis* mutant bearing the PsaB C565S change [56, 57] (see below, "Cluster interconversion"). A similar conclusion was reached when substituting the other suspected cysteine ligand of F_x by histidine in the PsaB subunit of the green alga *Chlamydomonas reinhardtii* photosystem I [58].

In contrast to the above complex, the structure and ligands of *Clostridium pasteurianum* 2[4Fe-4S] ferredoxin can be precisely inferred from the strong similarity with the homologues of known structure isolated from *Peptostreptococcus asaccharolyticus* [1, 2] or *Clostridium acidurici* [59]. Each cluster is held by binding motif 1 (see above, "[4Fe-4S] ferredoxins") with an added remote cysteine followed by a proline. The expression system implemented for this protein affords rapid screening of the stability of the modified forms [60–62]. The replacement of the second cysteine of the triplet (cysteine 11) by S, D, A, Q or P destabilizes the protein so much that no peptide can be detected in extracts. Similarly, when the C42L change is carried out in *Rhizobium meliloti* FdxN, expression of the corresponding gene does not restore the symbiotic nitrogen fixation activity of a mutant with a deleted *fdx*N gene [63].

When the remote cysteine (cysteine 18) associated with binding motif 1 is subjected to similar changes, the outcome is somewhat different. The C18Q and C18A forms could not be detected in the extracts, like in the case of the C11X peptides, but the polypeptide chains were clearly present for C18S, C18Y, C18H and C18D, in ascending order of concentration. None of these molecules were stable enough to be purified. However, extracts containing C18H could mediate electron transfer between the two major redox partners of this ferredoxin, pyruvate-ferredoxin oxidoreductase and hydrogenase, at a rate approximately 40 times slower than with native recombinant ferredoxin (Fig. 2) in a biological assay reproducing the *in vivo* electron transfer chain [64, 65]. Although no evidence about the nature of the prosthetic groups involved could be obtained, it is likely that some redox active component can be associated, at least transiently, with C18H. Similar experiments carried out with C18Q failed to restore H_2 evolution from pyruvate in the same conditions, hence confirming that the activity observed with C18H is indeed linked to the modified form of the ferredoxin.

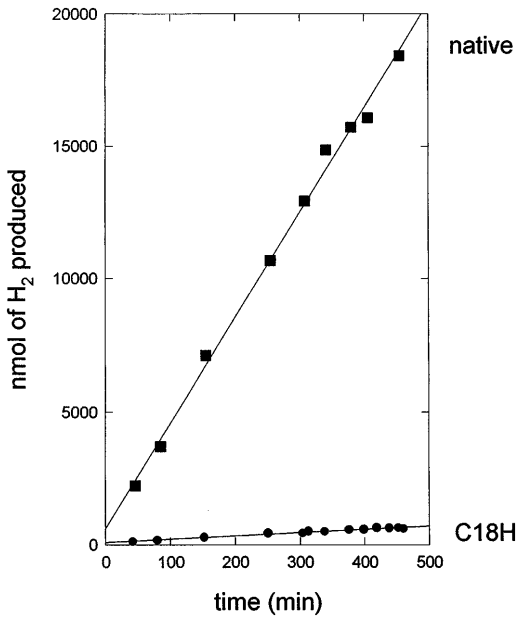

Fig. 2 Activity of the native and of the cysteine-18-substituted forms of *Clostridium pasteurianum* 2[4Fe-4S] ferredoxin as electron transfer agents. The activity was measured as the ability of the ferredoxin to couple pyruvate oxidation catalyzed by pyruvate-ferredoxin oxidoreductase to hydrogen evolution catalyzed by hydrogenase [64, 65]. The same amounts of *Escherichia coli* soluble extracts containing the recombinant ferredoxins were used for the native protein (*squares*) and for the C18H form (*circles*). The identical experiment carried out with cells containing the plasmid encoding C18Q did not yield any hydrogen production

These experiments with *Clostridium pasteurianum* 2[4Fe-4S] ferredoxin clearly show that detection of the polypeptide produced from a mutated gene might disclose the ability of the protein to be stabilized by the insertion of the cluster(s), at least in the case of small proteins. Therefore, care should be exercised when interpreting the results of SDM experiments on ligands of Fe-S proteins. It is not because a modified protein cannot be isolated with a full content of cofactor that the change introduced altogether impedes cluster assembly. The protein may, instead, be able to bind a functional core only transiently. Then measurements of the biological activity with marginally processed samples, when possible, are a useful source of information.

Cases in which no cluster is associated with the protein

Many SDM experiments carried out on presumptive Fe-S cluster ligands have resulted in the synthesis of

only the apoprotein of interest. The lack of cluster assembly in a modified protein may indeed be taken as an indication that the changed amino acid is directly bound to the inorganic cofactor in the wild-type protein.

For instance, many clues point to mixed Cys/His ligation for the Rieske-like proteins (see above, "Rieske protein"). Four cysteines and two histidines, conserved in all bc_1 complexes (C133, H135, C138, C153, C155 and H156 in the *Rhodobacter capsulatus* sequence), are candidate ligands. All of these residues have been individually replaced by various other amino acids [66–69], and in only one case (C155S; see below "[2Fe-2S] centers") was a [2Fe-2S] cluster assembled in the protein [68]. For the other forms generated, data concerning the presence of the aposubunit has varied depending on the study or on the source of protein [67–69]. The Rieske protein is one of the very few cases where SDM experiments carried out on potential ligands of the [2Fe-2S] cluster have not helped to resolve the issue. The strongest piece of evidence concerning the ligands of these proteins stems from sequence comparisons with the Rieske-like proteins of bacterial dioxygenases, which do not have the counterparts of C138 and C155 [43]. From this observation, the four remaining candidates (two Cys and two His) are inferred as the most likely ligands of the [2Fe-2S] cluster.

In adrenodoxin [70] and putidaredoxin [71], the [2Fe-2S] cluster was not inserted upon C→S substitutions of some of the cysteine residues, which were thereby identified as ligands.

Attempts have been made to assign the ligands of the [2Fe-2S] (center 1), [3Fe-4S] (center 3) and [4Fe-4S] (center 2) clusters of the Fe-S subunit (FrdB) of fumarate reductase. While the *Escherichia coli* strains having S or D instead of C65, a suspected ligand of center 1, could grow with fumarate or succinate as substrates (see below, "[2Fe-2S] centers"), those bearing the C65A form could not. This variant lacked the characteristic EPR spectrum of center 1, but not those of centers 2 and 3 [72]. It was concluded that C65 was a ligand of this [2Fe-2S] cluster.

Other cysteines of *Escherichia coli* fumarate reductase FrdB have been probed by SDM [73]. The three cysteines in positions 204, 210 and 214 were individually replaced by serines. These changes resulted in the loss of both centers 2 and 3 and in the failure of the FrdAB complex to associate with the membrane. Therefore, the identification of these ligands had to be deduced from additional experiments (see below, "Cluster interconversion").

In *Escherichia coli* nitrate reductase A, the β-subunit (NarH) contains three [4Fe-4S] clusters and one [3Fe-4S] cluster. The cysteines of the sequence are accordingly arranged in four groups of four with the generic spacing found in ferredoxins (binding motif 1), except that the third group has a tryptophan in place of the second cysteine. The substitution of the first cysteine of groups II–IV by alanine led to the loss of all Fe-S centers [74]. In contrast, individual replacements of the first two cysteines of group I by alanine or serine removed only the highest potential [4Fe-4S] center [75]. These thorough studies of a multicenter protein clearly show that the role of each Fe-S cluster in the folding of the recipient protein may be different despite the similar stretches of amino acid sequences binding them.

Azotobacter vinelandii hydrogenase contains two [4Fe-4S] clusters and one [3Fe-4S] cluster in its small subunit, like the enzyme from *Desulfovibrio gigas*. Other similarities, such as the sequence homology, between the two enzymes enable use of the recently determined structure of the *D. gigas* enzyme [36] to interpret the results of SDM experiments carried out on *A. vinelandii* hydrogenase [76, 77]. The individual substitution of two of the cysteine ligands of the [3Fe-4S] cluster by serines was found to abolish the activity [76], although highly variable H_2 evolution rates have recently been reported [77]. In contrast, similar experiments carried out on the cysteine-containing N-terminal motif that binds the proximal (to the Ni center) [4Fe-4S] cluster with all sulfur coordination [36] only decreased the activity of the enzyme [76, 77] (see below, "[4Fe-4S] centers").

The *A. vinelandii* Fe-protein of the nitrogenase complex is a homodimer with five conserved cysteines on each subunit as potential ligands for the sole [4Fe-4S] cluster bridging the subunits. Their replacement by serine residues led to the identification of the two that coordinate the cluster before the availability of the X-ray structure, since no incorporation of the Fe-S cluster occurred when the ligands were replaced [78].

In the glutamine phosphoribosylpyrophosphate aminotransferase from *Bacillus subtilis*, two C→S substitutions individually impeded the building of the Fe-S cluster, but, interestingly, the effect of one of them could be partly counterbalanced by the introduction of an additional cysteine [79, 80].

One of the most unexpected developments of research on aconitase has been the discovery of the identity between the cytoplasmic form of the enzyme and the protein binding to mRNA sequence elements, known as iron-responsive elements, to regulate iron homeostasis in eukaryotes [81]. The apo-form is the RNA binding protein and, under iron-replete conditions, the building of the [4Fe-4S] cluster converts the protein into active aconitase (see above, "Aconitase"). Substitution, by serine, of any of the three cysteines holding the cluster does not abolish mRNA binding but prevents the formation of the [4Fe-4S] cluster and therefore the onset of aconitase activity [82, 83].

The ligands of the P-cluster pair of nitrogenase can be arranged in two groups: one contains the singly coordinating cysteines from each subunit and one serine of the β-subunit, while the other includes the two cysteines bridging the Fe_4 halves of the pair [37–40]. For the cysteines of the first group, all replacements

have been found to suppress the assembly of the metal centers, except for the replacement by a serine of one cysteine of the β-subunit bound to the iron having another serine nearby [84–88]. For the bridging cysteines, only the substitution of the one contributed by the α-subunit was allowed [87].

All these examples show that the failure to incorporate any Fe-S cluster in a protein is a very likely event. This is true when the ligands of the cluster are changed, but also when other residues are replaced. Although such results are informative in terms of the factors required for the overall folding of Fe-S proteins, they are of relatively little value from the point of view of the bioinorganic chemist, since they generally do not reveal whether the problem is chemical (the cluster cannot swap coordinating groups) or biochemical (the protein conformation is no longer suited to coordinate the cluster). In order to alleviate the frustration of the patient bioinorganic chemist who has followed the discussion up to this point, those examples in which ligand substitutions have permitted identification of proteins coordinating a cluster will be presented below.

Cases in which a cluster is associated with the protein

In those cases where the incorporation of the Fe-S cluster is not impeded by SDM experiments on its ligands, either the cluster is converted into another one of different nuclearity, or the same cluster type is conserved with a partially renewed set of ligands.

Cluster interconversion

The best documented example of Fe-S interconversion is that between [3Fe-4S] and [4Fe-4S] clusters. Although other types of reactions, e.g. the [4Fe-4S] → [2Fe-2S] step in the degradation of the nitrogenase Fe-protein [89] and the formation of a linear trinuclear cluster in partially unfolded aconitase [90], have been described, only the interchange between [3Fe-4S] and [4Fe-4S] clusters has been reported as the result of SDM experiments on ligands. Interestingly, the latter conversion is a rather facile biochemical event that occurs in many proteins, but it is extremely difficult to reproduce with synthetic analogues [21].

In the FrdB subunit of *Escherichia coli* fumarate reductase, the replacement of valine 207 by cysteine introduced the genuine binding motif 1 of [4Fe-4S] clusters, and the produced protein was shown to contain a [4Fe-4S] cluster instead of the native [3Fe-4S] cluster 3 [73]. Conversely, many examples of [4Fe-4S] → [3Fe-4S] transformations appeared as the result of cysteine substitutions. This was the case for at least part of the population of the PsaB protein of photosystem I when serine replaced cysteine 565 [56]. A similar result occurred in DMSO reductase DmsB when C102 was substituted by tryptophan, serine, tyrosine or phenylalanine [91] and in protein PsaC of photosystem I when either cysteine 14 or cysteine 51 was replaced by aspartate [92, 93]. In these latter cases, the SDM experiments mimicked the absence of the second cysteine of binding motif 1 in some genuine [3Fe-4S] proteins. However, the substitution of the cysteine in this position is not a factor which, by itself, prevents the building of a [4Fe-4S] center [94] (see above, "[4Fe-4S] ferredoxins"), and similar amino acid replacements may lead to different results in other systems, as exemplified below.

Ligand exchange

[1Fe] centers. The active site of rubredoxin is unusual in that it does not contain inorganic sulfur and it is the only example in which a single iron ion has a complete tetrahedral coordination of sulfurs provided by cysteines. Indeed, a ferrous/ferric ion can be coordinated by virtually any set of ligands, as shown by many examples of metalloproteins ([95] and references therein). An all-sulfur (rubredoxin-like) site is even known to coexist with a high-spin iron site displaying octahedral coordination contributed by mainly oxygen or nitrogen ligands in desulfoferrodoxin [96].

Therefore, an iron atom with non-sulfur ligands is not a rare occurrence, but very few studies have been aimed at changing one of the ligands of a complete sulfur coordination sphere in rubredoxin. It is worth mentioning that coordination by cysteines in rubredoxins stabilizes tetrahedral geometry, while the involvement of other functional groups in other Fe-proteins favors penta- or, more often, hexa-coordination. The replacement of either cysteine 6 or cysteine 9 of *Clostridium pasteurianum* rubredoxin by aspartate induces significant spectroscopic changes associated with the chromophore [97]. Upon substitution of cysteine 42 of the same rubredoxin by serine [98], the spectroscopic properties of the active site are also altered. These data are all consistent with the coordination of one aspartate (probably monodentate) or one serine to the iron, and the available evidence suggests that the coordination number of the iron has remained unchanged. However, the two types of derivatives (C9D, C42S) exhibit redox potentials differing by ca. 250 mV [97, 98]. No rationale exists for this discrepancy, the elucidation of which should deepen our understanding both of the coordination chemistry of iron in this case and of the factors that tune the value of the reduction potential in rubredoxins.

[2Fe-2S] centers. [2Fe-2S] proteins provide the largest data set of Fe-S proteins for which the replacements of cysteine ligands by serine, and in a few instances by alanine or aspartate, have permitted assembly of the cluster. Such experiments were performed on the Rieske subunit of *Rhodobacter capsulatus* bc_1 complex [68]

(see above, "Cases in which no cluster is associated with the protein"), on the FrdB subunit of *Escherichia coli* fumarate reductase [72, 99], on the [2Fe-2S] ferredoxin from *C. pasteurianum* [100, 101], on the vegetative cell ferredoxin from *Anabaena* sp. 7120 [102], and on the NQO2 subunit of *Paracoccus denitrificans* NADH-quinone oxidoreductase [103]. The main goals of these studies were the identification of the ligands, in cases where they were unknown, and the engineering of Fe-S clusters with new coordination spheres.

When cysteine ligands were replaced by serine, the results were strongly dependent on the target protein and on the position of the cysteine residue. Serinate binding could be assessed in some cases by shifts to higher energy of electronic absorption transitions [100, 102], by upshifts of some resonance Raman bands [101], by increases in the rhombicity of EPR spectra, often with concomitant decreases of the average g value [100, 102], and, apparently in one case, by crystal structure [102]. Reduction potentials were shifted, in many cases [99] (S.E.J. Fawcett, M.-P. Golinelli, J. Meyer and F.A. Armstrong, unpublished data), to values at least 100 mV more negative, in agreement with observations made upon analogous substitutions in synthetic model compounds ([104] and references therein). Smaller shifts of variable sign were also observed [99, 102], one significant exception being the ca. 100-mV positive shift for the C49S mutant of *Anabaena* ferredoxin [102]. In the FrdB subunit [99] and in the *C. pasteurianum* [2Fe-2S] ferredoxin [101] (S.E.J. Fawcett, M.-P. Golinelli, J. Meyer and F.A. Armstrong, unpublished data), good correlation was found between large negative potential shifts and increases in the rhombicity of the EPR spectra, which suggests that in these cases the serinate ligand is bound to the redox active iron. C79S of *Anabaena* ferredoxin [102] and C11S of *C. pasteurianum* ferredoxin [101] were so unstable that their thorough characterization was impeded. Their instability alone agrees with the substituted cysteine being a ligand of the [2Fe-2S] cluster. In those cases where structural data are available, no correlations have been found between the stability of the variants and either the number of hydrogen bonds involving the sulfur atom of the replaced cysteine or the conformation of this ligand.

Somewhat puzzling was the observation of similar properties for proteins in which cysteines were replaced by either serine or alanine: this was the case for *C. pasteurianum* ferredoxin C24S and C24A [101] and for all the cysteines of the NQO2 subunit [103]. Whereas such results strongly suggest that serinate is not a ligand in these cases, the actual ligands of the [2Fe-2S] cluster in these variants remain to be identified. Recent data suggest that C14 can act as a substitute for C24 in *C. pasteurianum* ferredoxin (M.-P. Golinelli and J. Meyer, unpublished data), a situation reminiscent of that demonstrated with the C20A form of *Azotobacter vinelandii* ferredoxin I [105–107] (see below, "[4Fe-4S] centers"). Analogous exchanges of cysteines have been invoked in the case of the NQO2 subunit [103], but their occurrence for all ligands appears unlikely. Coordination by solvent molecules is another possibility in the case of at least some of the NQO2 variants and for the doubly substituted C14A/C24A form of *C. pasteurianum* ferredoxin [101], but experimental evidence remains to be obtained. Replacement of cysteine ligands by alanine prevented the assembly of the Fe-S cluster in the C65A form of FrdB [72] (see above, "Cases in which no cluster is associated with the protein"), and in the C56A *C. pasteurianum* ferredoxin (M.-P. Golinelli and J. Meyer, unpublished data), presumably because no substitute ligand could have access to these coordination sites.

The C56H, C56D and C56N variants of *C. pasteurianum* ferredoxin contain very low amounts of unstable chromophore and yield intermediate results between complete lack of cluster assembly and involvement of another set of ligands (J. Meyer and J. Gaillard, unpublished data).

It should be mentioned that the destabilizing effects induced by each ligand substitution are at least additive in the cases of multiple replacements. For instance, no chromophore is assembled in the C56S/C60S form of *C. pasteurianum* ferredoxin (M.-P. Golinelli and J. Meyer, unpublished data), whereas the singly substituted variants are relatively stable [100, 101]. This observation suggests that [2Fe-2S] clusters with more than one serinate ligand are unlikely to be assembled in any protein.

The different results obtained with C→S/A substitutions in [2Fe-2S] proteins indicate that these effects arise from several causes. These may include solvent access to the coordination site, the presence of neighboring functional residues that might act as substitutes for the cysteine ligand, and the flexibility of the polypeptide chain which will have to accommodate a *ca.* 0.5-Å shortening of the metal-ligand bond upon replacement of cysteine by serine [18, 102]. The localization of the Fe(II) valence is also an important parameter. In the wild-type proteins, environmental factors contributed by the polypeptide chain are likely to determine which of the two irons is more reducible. Upon replacement of one of the cysteine ligands by serine, the electron localization may be preserved [102] or shifted to the other iron atom. Recently, double exchange within a valence-delocalized [2Fe-2S] cluster has been proposed to be responsible for the $S = 9/2$ ground state observed in the C60S variant of *C. pasteurianum* ferredoxin [108]. The introduction of a serinate ligand in this case might then cancel out some of the environmental differences between the two iron atoms.

[4Fe-4S] centers. Besides the ligands of its [3Fe-4S] and [4Fe-4S] clusters, *Azotobacter vinelandii* ferredoxin I has an additional free cysteine residue in position 24. When cysteine 20, which is the remote residue of the [4Fe-4S] binding motif, was replaced by alanine, it was

Table 1 Consequences of substituting ligands of Fe-S clusters in protein (*asterisk* result of substitution, *question mark* suspected but unproven result)

Protein	Source	Cluster type	Substitution	No peptide	Apo-protein	Cluster conversion	Ligand exchange	References
PsaB	*Synechocystis* sp. PCC 6803	[4Fe-4S] (F_x)	C565H	*				[55]
			C565D	*				
			C565S			*	*	[56]
			C556S + C565S				*	[57]
	Chlamydomonas reinhardtii	[4Fe-4S] (F_x)	C560H	*				[58]
2[4Fe-4S] ferredoxin	*Clostridium pasteurianum*	[4Fe-4S]	C11S,D,A	*				[110]
			C11Q,P	*				
			C18A,Q	*				[109]
			C18S,Y,H,D		*		?	
	Rhizobium meliloti	[4Fe-4S]	C42L	?				[63]
[2Fe-2S] Rieske protein	*Saccharomyces cerevisiae*	[2Fe-2S]	C148Y	*				[66]
			C159S, C164S, C178S, C180S, H161R, H181R		*			[67]
	Rhodobacter capsulatus		C133R,S	*				[68]
			C138R,S,F		*			
			C153R,S		*			
			C155G,D		*			
			H135L,P	*				
			H156L,P,F,T,Y		*			
			C155S				?	
	Rhodobacter sphaeroides		C129G, C134S, C149Y		?			[69]
			H131C, C151S		*			
Adrenodoxin	Adrenal cortex	[2Fe-2S]	C46S, C52S, C55S, C92S		*			[70]
Putidaredoxin	*Pseudomonas putida*	[2Fe-2S]	C86S	*				[71]
FrdB	*Escherichia coli*	[2Fe-2S]	C65A		a			[72]
			C65S,D				*	[72, 99]
			C57S, C62S, C77S					
		[3Fe-4S]	C204S, C210S		a			[73]
			V207C			*		
		[4Fe-4S]	C214S		a			
NarH	*Escherichia coli*	[3/4Fe-4S]	C184A, C217A, C244A		*			[74]
		[4Fe-4S]	C184S				?	
		[4Fe-4S]	C16A,S, C19A,S		a			[75]
DmsB	*Escherichia coli*	[4Fe-4S]	C102W,S,Y,F			*		[91]

shown, most clearly by X-ray crystallography, that formation of the same cluster could recruit the free cysteine 24 as a substitute ligand [105–107]. A similar situation may hold in the case of *Azotobacter vinelandii* hydrogenase, where the two cysteines 62 and 65 binding the [4Fe-4S] cluster close to the Ni site were replaced by serines [76]. Decreased activity was detected, suggesting that the additional cysteine 64 may substitute for the replaced ligand. However, the idea is not sustained by a recent reevaluation of the activity of an enzyme in which cysteine 64 was replaced by serine as well [77]. A related observation was made with the glutamine phosphoribosylpyrophosphate aminotransferase from *Bacillus subtilis* when the deleterious effect of one C→S substitution was cancelled out by a nearby D→C change [79, 80] (see above, "Cases in which no cluster is associated with the protein").

A different situation can be invoked in the case of the C184S form of *Escherichia coli* NarH [74]. Almost all substitutions involving ligands of the Fe-S clusters impaired the insertion of the prosthetic group in this nitrate reductase [74, 75] (see above, "Cases in which no cluster. . ."): this included the C184A replacement, but not the C184S one which decreased the activity without a clear alteration of the set of Fe-S clusters. A serine ligand for one [4Fe-4S] cluster in this case is still a tempting working hypothesis, but the definite involvement of a serinate in the coordination sphere of a protein-bound [4Fe-4S] cluster remains to be established. Another example is provided by the PsaC pro-

Table 1 (continued)

Protein	Source	Cluster type	Substitution	No peptide	Apo-protein	Cluster conversion	Ligand exchange	References
Nitrogenase Fe-protein	*Azotobacter vinelandii*	[4Fe-4S]	C97S, C132S		*			[78]
PurF	*Bacillus subtilis*	[4Fe-4S]	C448S, C451S		*			[79, 80]
			C451S + D442C				?	
Aconitase/ IRE-BP	Chimeric human mouse	[4Fe-4S]	C437S, C503S, C506S		*			[82]
	Human		C437S, C503S, C506S		*			[83]
Hydrogenase	*Azotobacter vinelandii*	[3Fe-4S]	C294S, C297S				?	[76, 77]
		[4Fe-4S]	C62S, C65S				?	
			C64S + C65S				?	
PsaC	*Synechococcus* sp. PCC 7002	[4Fe-4S]	C14D, C51D			*		[92–94]
			C14D				*	
Rubredoxin	*Clostridium pasteurianum*	[1Fe]	C6D, C9D				*	[97]
			C42S				*	[98]
Nitrogenase Mo-Fe protein	*Azotobacter vinelandii*	P-cluster	αC62A,S,K,V,D,L,G,E		*			[84, 87, 88]
			αC154A,S,E,Q,N,T		*			
			αC88A,Q,P,V,R		*			
			αC88S,G,T,D				?	
			βC70A,S		*			
			βC153A		*			
			βC95S		*			
			βC153S				?	
	Klebsiella pneumoniae		αC63A, αC155A,S		*			[85, 86]
			αC89A		*			
			βC69A, βC152A		*			
			βC94A		*			
			βC152S				?	
[2Fe-2S] ferredoxin	*Clostridium pasteurianum*	[2Fe-2S]	C11S, C56S, C60S				*	[100, 101]
			C24S, C24A,				*	
			C14A + C24A				*	
			C56S + C60S		*			Unpublished data from M.-P. Golinelli, J. Gaillard and J. Meyer
			C56H, C56D, C56N				?	
			C56A		*			
[2Fe-2S] ferredoxin	*Anabaena* sp. 7120	[2Fe-2S]	C41S, C46S, C49S, C79S				*	[102]
NQO2	*Paracoccus denitrificans*	[2Fe-2S]	C79A,S, C101A,S, C137A,S, C141A,S				*	[103]
Ferredoxin I	*Azotobacter vinelandii*	[4Fe-4S]	C20A				*	[105–107]

[a] The protein is detected with only part of its clusters

tein of photosystem I, in which the C14D substitution results in a mixed-ligand [4Fe-4S] cluster when the subunit is associated with the rest of the photosystem I complex [94].

Centers of higher nuclearity. Most substitutions involving the ligands of the P-cluster pair of nitrogenase lead to the loss of both the nitrogen fixation activity and the cofactors of the Mo-Fe protein [84–88]. However, of the two cysteines bridging the Fe_4S_4 units of the P-cluster pair, the one contributed by subunit α could be efficiently replaced by threonine and aspartate, less so by glycine and serine, and not at all by alanine, glutamine, proline, valine or arginine [87]. It may be that the presence of a bridging ligand is not mandatory in this position to afford an active cluster; this is not true, however, for the bridging cysteine ligand contributed by the β-subunit, the substitution of which cancels both activity and cluster assembly [85, 87, 88]. For the monodentate ligands of the P-cluster, the cysteine of subunit β

coordinating the iron ion, in the vicinity of which a serine is also found (see above, "Nitrogenase"), can apparently be replaced by a serine [85, 88]; this change decreases the intramolecular electron flux between the P-clusters and the FeMo cofactor [88]. Since this residue is the only one among the terminal ligands of the P-cluster to stand substitution by serine, one may infer that interactions of the corresponding iron with the nearby serinates can mimic, in part, those occurring with the native ligands.

Conclusions

From the published data dealing with the substitution of the ligands of Fe-S proteins, it seems that the chances of creating molecules with mixed-type ligands are slim (Table 1), especially compared with similar studies carried out with synthetic analogues (see above, "Ligand exchange on synthetic compounds"). In many cases, substituting only one ligand results in complete loss of the cluster(s). From the relatively limited data set available, such occurrences seem unpredictable; however, several factors, such as the intrinsic instability of the native cluster, that of the holoprotein, the easy access of the solvent to the Fe-S center and the role of the cluster as a bridge between subunits, may be considered as detrimental. In this respect, targeting of primary structure positions, found interesting in some studies, does not guarantee success with other systems.

For those studies that have afforded an active center in the presence of a non-cysteine ligand, only the simplest Fe-S centers of rubredoxins and [2Fe-2S] proteins seem to easily accept serinyl or carboxyl coordination. The ultimate proof of the formation of a Fe-O bond has not yet been published but should soon appear for at least some of these proteins [102].

In the cases of clusters with more than three iron atoms, the removal of a native ligand leads to consequences different from those observed with rubredoxins or [2Fe-2S] proteins. In such instances, it appears that the modified protein tends to devise means of either changing the cluster's nuclearity or finding alternate thiolates in order to avoid using a different functional group. It may be that, in contrast to what has been established with synthetic compounds, the stability and properties of Fe_mS_n ($m>2$) clusters in proteins rely heavily on the nature of their ligands. Only in specific cases, e.g. aconitase, can non-sulfur coordination be accepted and even beneficial. The possibility of replacing ligands in Fe-S proteins may thus be the result of a delicate balance between the versatility of the cluster's chemistry and the flexibility of the surrounding environment, in particular of the polypeptide stretches bearing the ligands.

In view of the largely unexpected results obtained so far from SDM experiments involving the ligands of Fe-S centers, there seems to be little doubt that future work along these lines will enlarge our understanding of these proteins and disclose new frontiers for this field of research.

Acknowledgements We thank Professor G. La Mar for providing unpublished material and Professor D. Rees for clarifying discussions.

References

1. Adman ET, Sieker LC, Jensen LH (1973) J Biol Chem 248:3987–3996
2. Adman ET, Sieker LC, Jensen LH (1976) J Biol Chem 251:3801–3806
3. Carter CW Jr, Kraut J, Freer ST, Xuong N-H, Alden RA, Bartsch RG (1974) J Biol Chem 249:4212–4225
4. Freer ST, Alden RA, Carter CW Jr, Kraut J (1975) J Biol Chem 250:46–54
5. Watenpaugh KD, Sieker LC, Herriot JR, Jensen LH (1973) Acta Crystallogr B29:943–956
6. Watenpaugh KD, Sieker LC, Jensen LH (1980) J Mol Biol 138:615–633
7. Tsukihara T, Fukuyama K, Tahara H, Katsube Y, Matsuura Y, Tanaka N, Kakudo M, Wada K, Matsubara H (1978) J Biochem 84:1645–1647
8. Fukuyama K, Hase T, Matsumoto S, Tsukihara T, Katsube Y, Tanaka N, Kakudo M, Wada K, Matsubara H (1980) Nature 286:522–524
9. Adman ET, Watenpaugh KD, Jensen LH (1975) Proc Natl Acad Sci USA 72:4854–4858
10. Dalton H, Mortenson LE (1972) Bacteriol Rev 36:231–260
11. Raeburn S, Rabinowitz JC (1971) Arch Biochem Biophys 146:21–33
12. Cammack R (1992) Adv Inorg Chem 38:281–322
13. Johnson MK (1994) In: King RC (ed) Encyclopedia of inorganic chemistry. Wiley, Chichester, UK, pp 1896–1915
14. Holm RH (1977) Acc Chem Res 10:427–434
15. Herskovitz T, Averill BA, Holm RH, Ibers JA, Phillips WD, Weiher JF (1972) Proc Natl Acad Sci USA 69:2437–2441
16. Beardwood P, Gibson JF (1992) J Chem Soc Dalton Trans pp. 2457–2466
17. Salifoglou A, Simopoulos A, Kostikas A, Dunham RW, Kanatzidis MG, Coucouvanis D (1988) Inorg Chem 27:3394–3406
18. Cleland WE, Holtman DA, Sabat M, Ibers JA, DeFotis GC, Averill BA (1984) J Am Chem Soc 105:6021–6031
19. Johnson RE, Papaefthymiou GC, Frankel RB, Holm RH (1983) J Am Chem Soc 105:7280–7287
20. Evans DJ, Leigh GJ (1991) J Inorg Biochem 42:25–35
21. Holm RH (1992) Adv Inorg Chem 38:1–71
22. Hong JS, Rabinowitz JC (1970) J Biol Chem 245:6582–6587
23. Moulis J-M, Meyer J (1982) Biochemistry 21:4762–4771
24. Reynolds JG, Holm RH (1981) Inorg Chem 20:1873–1878
25. Que L Jr, Bobrick MA, Ibers JA, Holm RH (1974) J Am Chem Soc 96:4168–4178
26. Maskiewicz R, Bruice TC (1977) Biochemistry 16:3024–3029
27. Kennedy MC, Beinert H (1988) J Biol Chem 263:8194–8198
28. Hurley JK, Caffrey MS, Markley JL, Cheng H, Xia B, Chae YK, Holden HM, Tollin G (1995) Protein Sci 4:58–64
29. Beinert H, Kennedy M-C (1989) Eur J Biochem 186:5–15
30. Kennedy MC, Stout CD (1992) Adv Inorg Chem 38:323–339
31. Robbins AH, Stout CD (1989) Proc Natl Acad Sci USA 86:3639–3643
32. Robbins AH, Stout CD (1989) Proteins Struct Funct Genet 5:289–312

33. Lauble H, Kennedy MC, Beinert H, Stout CD (1992) Biochemistry 31:2735–2748
34. Lauble H, Kennedy MC, Beinert H, Stout CD (1994) J Mol Biol 238:437–451
35. Lauble H, Stout CD (1995) Proteins Struct Funct Genet 22:1–11
36. Volbeda A, Charon M-H, Piras C, Hatchikian EC, Frey M, Fontecilla-Camps JC (1995) Nature 373:580–587
37. Chan MK, Kim J, Rees DC (1993) Science 260:792–794
38. Kim J, Rees DC (1992) Science 257:1677–1682
39. Kim J, Rees DC (1992) Nature 360:553–560
40. Kim J, Woo D, Rees DC (1993) Biochemistry 32:7104–7115
41. Kim J, Rees DC (1994) Biochemistry 33:389–397
42. Trumpower BL (1990) Microbiol Rev 54:101–129
43. Mason JR, Cammack R (1992) Annu Rev Microbiol 46:277–305
44. Beinert H, Thomson AJ (1983) Arch Biochem Biophys 222:333–361
45. Conover RC, Park J-B, Adams MWW, Johnson MK (1991) J Am Chem Soc 113:2799–2800
46. Butt JN, Sucheta A, Armstrong FA, Breton J, Thomson AJ, Hatchikian EC (1993) J Am Chem Soc 115:1413–1421
47. Park J-B, Fan C, Hoffman BM, Adams MWW (1991) J Biol Chem 266:19351–19356
48. George SJ, Armstrong FA, Hatchikian EC, Thomson AJ (1989) Biochem J 264:275–284
49. Calzolai L, Gorst CM, Zhao Z-H, Teng Q, Adams MWW, La Mar GN (1995) Biochemistry 34:11373–11384
50. Shen B, Martin LL, Butt JN, Armstrong FA, Stout CD, Jensen GM, Stephens PJ, La Mar GN, Gorst CM, Burgess BK (1993) J Biol Chem 268:25928–25939
51. Flint DH, Emptage MH, Finnegan MG, Fu W, Johnson MK (1993) J Biol Chem 268:14732–14742
52. Darlinson MG, Guest JR (1984) Biochem J 223:507–517
53. Meyer J, Gagnon J (1991) Biochemistry 30:9697–9704
54. Pierik AJ, Hagen WR, Dunham WR, Sands RH (1992) Eur J Biochem 206:705–719
55. Smart LB, Warren PV, Golbeck JH, McIntosh L (1993) Proc Natl Acad Sci USA 90:1132–1136
56. Warren PV, Smart LB, McIntosh L, Golbeck JH (1993) Biochemistry 32:4411–4419
57. Vassiliev IR, Jung Y-S, Smart LB, Schulz R, McIntosh L, Golbeck JH (1995) Biophys J 69:1544–1553
58. Webber AN, Gibbs PB, Ward JB, Bingham SE (1993) J Biol Chem 268:12990–12995
59. Duée ED, Fanchon E, Vicat J, Sieker LC, Meyer J, Moulis J-M (1994) J Mol Biol 243:683–695
60. Davasse V, Moulis J-M (1992) Biochem Biophys Res Commun 185:341–349
61. Moulis J-M, Davasse V, DeJésus F (1994) Biometals 7:272–278
62. Davasse V, Quinkal I, Gaillard J, Moulis J-M (1995) In: Geisow M, Epton R (eds) Perspectives on protein engineering. Mayflower Worldwide, Birmingham, UK, pp 152–155
63. Masepohl B, Kutsche M, Riedel K-U, Schmehl M, Klipp W, Pühler A (1992) Mol Gen Genet 233:33–41
64. Quinkal I, Davasse V, Gaillard J, Moulis J-M (1994) Protein Eng 7:681–687
65. Moulis J-M, Davasse V (1996) Biochemistry (in press)
66. Gatti DL, Meinhardt SW, Ohnishi T, Tzagoloff A (1989) J Mol Biol 205:421–435
67. Graham LA, Trumpower BL (1991) J Biol Chem 266:22485–22492
68. Davidson E, Ohnishi T, Atta-Asafo-Adjei E, Daldal F (1992) Biochemistry 31:3342–3351
69. Van Doren SR, Gennis RB, Barquera B, Crofts AR (1993) Biochemistry 32:8083–8091
70. Uhlmann H, Beckert V, Schwarz D, Bernhardt R (1992) Biochem Biophys Res Commun 188:1131–1138
71. Gerber NC, Horiuchi T, Koga H, Sligar SG (1990) Biochem Biophys Res Commun 169:1016–1020
72. Werth MT, Sices H, Cecchini G, Schröder I, Lasage S, Gunsalus RP, Johnson MK (1992) FEBS Lett 299:1–4
73. Manodori A, Cecchini G, Schröder I, Gunsalus RP, Werth MT, Johnson MK (1992) Biochemistry 31:2703–2712
74. Augier V, Guigliarelli B, Asso M, Bertrand P, Frixon C, Giordano G, Chippaux M, Blasco F (1993) Biochemistry 32:2013–3023
75. Augier V, Asso M, Guigliarelli B, More C, Bertrand P, Santini C-L, Blasco F, Chippaux M, Giordano G (1993) Biochemistry 32:5099–5108
76. Sayavedra-Soto LA, Arp DJ (1993) J Bacteriol 175:3414–3421. Erratum (1993) J Bacteriol 175:5744
77. McTavish H, Sayavedra-Soto LA, Arp DJ (1995) J Bacteriol 177:3960–3964
78. Howard JB, Davis R, Moldenhauer B, Cash VL, Dean DR (1989) J Biol Chem 264:11270–11274
79. Makaroff CA, Paluh JL, Zalkin H (1986) J Biol Chem 261:11416–11423
80. Grandoni JA, Switzer RL, Makaroff CA, Zalkin H (1989) J Biol Chem 264:6058–6064
81. Beinert H, Kennedy MC (1993) FASEB J 7:1442–1449
82. Philpott CC, Haile D, Rouault TA, Klausner RD (1993) J Biol Chem 268:17655–17658
83. Hirling H, Henderson BR, Kühn LC (1994) EMBO J 13:453–461
84. Brigle KE, Setterquist RA, Dean DR, Cantwell JS, Weiss MC, Newton WE (1987) Proc Natl Acad Sci USA 84:7066–7069
85. Kent HM, Ioannidis I, Gormal C, Smith BE, Buck M (1989) Biochem J 264:257–264
86. Kent HM, Baines M, Gormal C, Smith BE, Buck M (1990) Molecular Microbiol 4:1497–1504
87. Dean DN, Setterquist RA, Brigle KE, Scott DJ, Laird NF, Newton WE (1990) Molecular Microbiol 4:1505–1512
88. May HD, Dean DR, Newton WE (1991) Biochem J 277:457–464
89. Anderson GL, Howard JB (1984) Biochemistry 23:2118–2122
90. Kennedy MC, Kent TA, Emptage M, Merkle H, Beinert H, Münck E (1984) J Biol Chem 259:14463–14471
91. Rothery RA, Weiner JH (1991) Biochemistry 30:8296–8305
92. Zhao J, Li N, Warren P, Golbeck JH, Bryant DA (1992) Biochemistry 31:5093–5099
93. Yu L, Zhao J, Lu W, Bryant DA, Golbeck JH (1993) Biochemistry 32:8251–8258
94. Yu L, Bryant DA, Golbeck JH (1995) Biochemistry 34:7861–7868
95. Ohlendorf DH, Orville AM, Lipscomb JD (1994) J Mol Biol 244:586–608
96. Tavares P, Ravi N, Moura JJG, LeGall J, Huang Y-H, Crouse BR, Johnson MK, Huynh BH, Moura I (1994) J Biol Chem 269:10504–10510
97. O'Dell SE, Kurtz DM Jr, Scott RA, Smith ET (1994) J. Inorg. Biochem 56:18
98. Meyer J, Gaillard J, Lutz M (1995) Biochem Biophys Res Commun 212:827–833
99. Werth MT, Cecchini G, Manodori A, Ackrell BAC, Schröder I, Gunsalus RP, Johnson MK (1990) Proc Natl Acad Sci USA 87:8965–8969
100. Fujinaga J, Gaillard J, Meyer J (1993) Biochem Biophys Res Commun 194:104–111
101. Meyer J, Fujinaga J, Gaillard J, Lutz M (1994) Biochemistry 33:13642–13650
102. Cheng H, Xia B, Reed GH, Markley JL (1994) Biochemistry 33:3155–3164
103. Yano T, Sled VD, Ohnishi T, Yagi T (1994) FEBS Lett 354:160–164
104. Cleland WE Jr, Averill BA (1984) Inorg Chem 23:4192–4197

105. Martín AE, Burgess BK, Stout CD, Cash VL, Dean DR, Jensen GM, Stephens PJ (1990) Proc Natl Acad Sci USA 87:598–602
106. Soman J, Iismaa S, Stout CD (1991) J Biol Chem 266:21558–21562
107. Iismaa SE, Vázquez AE, Jensen GM, Stephens PJ, Butt JN, Armstrong FA, Burgess BK (1991) J Biol Chem 266:21563–21571
108. Crouse BR, Meyer J, Johnson MK (1995) J Am Chem Soc 117:9612–9613
109. Davasse V (1993) PhD thesis, Université Joseph-Fourier, Grenoble, France
110. Quinkal I (1995) PhD thesis, Université Joseph-Fourier, Grenoble, France
111. Kowal AT, Werth MT, Manodori A, Cecchini G, Schröder I, Gunsalus RP, Johnson MK (1995) Biochemistry 34:12284–12293
112. Babini E, Bertini I, Borsari M, Capozzi F, Dikiy A, Eltis L, Luchinat C (1996) J Am Chem Soc (in press)
113. Shen B, Jollie DR, Diller TC, Stout CD, Stephens PJ, Burgess BK (1995) Proc Natl Acad Sci USA 92:10064–10068

Note added in proof. The occurrence of serine coordination for protein-bound [4Fe-4S] clusters (see above, "[4Fe-4S] centers") has very recently been extended to new cases [111, 112]. However, the X-ray structure of the C20S form of *Azotobacter vinelandii* ferredoxin I [113] confirms that a local rearrangement to recruit a free cysteine as a substitute ligand may sometimes be preferred over simple ligand replacement of the native cysteine by serine.