Structure, function and evolution of bacterial ferredoxins

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1. INTRODUCTION

During the past few years, the field of iron-sulfur proteins has not ceased expanding. This evolution is due to the development of sophisticated methods such as electron paramagnetic resonance, Mössbauer or X-ray crystallography and to a fruitful interdisciplinary collaboration between biochemists, chemists and physicists. These advances have brought to light how widespread the non-heme iron proteins are, and their importance amongst the electron carrier proteins together with the flavoproteins, cytochromes and cuproproteins.

With some exceptions, all organisms are dependent upon transducing electron transport chains for the production of ATP. The two metals, iron and copper, naturally selected during biological evolution, have two easily accessible oxidation states. The iron-sulfur clusters are among the simplest electron transfer groups and have been suggested as the first to have been produced during chemical evolution [1]. Berg and Holm [2] have reported that (4Fe-4S) clusters assemble themselves from a mixture of iron, sulfide and thiol of cysteine peptide, under anaerobic conditions similar to those which existed on the primitive Earth. The origin and development of iron-sulfur proteins occurred in the prokaryotes and especially in anaerobic bacteria which contain ferredoxins characterized by a low molecular weight (6000) and two (4Fe-4S) clusters. This led to a diverse range of proteins with various combinations of (4Fe-4S) and (3Fe-3S) clusters, or with the addition of other prosthetic groups or metal ions in the cases of so-called conjugated (Fe-S) proteins. Iron-sulfur proteins are ubiquitous electron transport proteins, covering a wide range of functions from steroid hydroxylation, and CO₂ and H₂ fixation, to oxidative phosphorylation and photosynthesis. Structurally, there are, to date, several main types of iron-sulfur proteins, namely rubredoxins, (2Fe-2S), (3Fe-3S) and (4Fe-4S) type ferredoxins. The properties of these different groups of proteins have been extensively reported in several volumes and reviews [3–8]. The content of this present review will focus on bacterial ferredoxins and on the recent developments in this field.
2. DEFINITION, CLASSIFICATION AND GENERAL PROPERTIES OF FERREDOXINS

The ferredoxins group includes all proteins with non-heme iron in the active center which is non-covalently associated with acid-labile sulfide and cysteinyl sulfur. They are divided into several major groups on the basis of their iron and acid-labile contents (Fig. 1), redox properties and g values of their EPR spectra. The name ferredoxin, which literally means iron redox protein, was given by Mortenson et al. in 1962 [9] to a brownish protein isolated from Clostridium pasteurianum. Other proteins, isolated from anaerobic bacteria and plants which have chemical similarities and could be functionally interchangeable, were classified in the same group.

Their main characteristics are:
- the ability to transfer electrons in biological reactions
- the presence of non-heme iron and labile sulfur in equivalent amounts
- a molecular weight of the soluble protein of around 6000–13000
- the preponderance of acidic over basic residues in amino acid composition
- a low oxidation reduction potential
- a characteristic absorption spectrum in the visible region which decreases reversibly on treatment with physiological or artificial reducing systems
- an EPR signal in the oxidized or reduced states at low temperatures, characteristic of the cluster type.

As they are highly acidic, they are purified by adsorption on anion exchangers such as DEAE-cellulose [10]. Most ferredoxins are desorbed from DEAE-cellulose using a chloride gradient (0.3–0.4 M Cl⁻ at pH 7). Because of their low molecular weight, they are separated from high molecular weight proteins and nucleic acids by gel filtration on Sephadex G75 columns or Bio-Gel P10. Further purification is achieved by ammonium sulfate precipitation and calcium phosphate gel chromatography. Purification is carried out near 0 °C using buffers flushed with nitrogen or argon to avoid dissolved oxygen, as ferredoxin is very sensitive to oxygen and heat denaturation. Purity is followed with the absorption spectrum using the coefficient $A_{390}/A_{305}$.

2.1. The eight-iron ferredoxins

This class of ferredoxins is, so far, found only in bacteria, mainly in anaerobic fermenters like Clostridia, Peptococci, sulfate-reducing bacteria, but also in photosynthetic bacteria (Chlorobium, Chromatium and Rhodospirillum). They play an important role in the metabolism of anaerobic bacteria and can be reduced by hydrogenase. The reduction process is a change from oxidation level +2, corresponding to a (2Fe³⁺ 2Fe²⁺) cluster, EPR silent, to an oxidation level +1, corresponding to a (Fe³⁺ 3Fe²⁺) cluster. The latter state gives an EPR spectrum around $g = 1.96$. The feature of the EPR spectrum is characteristic of a system of clusters, close enough to interact magnetically. Their midpoint potential is about −400 mV. Their amino acid sequence is characterized by the presence of eight cysteine residues involved in the binding of the two (4Fe-4S) clusters, and the absence or a very low content of lysine, histidine, arginine, tryptophan and methionine.

After removing the iron and sulfur atoms from the native protein, the reconstitution of the active protein can be obtained by addition of iron and sulfide in a reducing medium [11,12].

2.2. The four-iron ferredoxins

These ferredoxins are very similar to the eight-iron ferredoxins in absorption spectra and redox properties. The EPR spectra are more simplified...
by the absence of magnetic interaction associated with the loss of one cluster.

The disappearance of the second cluster has to be correlated to the presence of only four or six cysteines in the protein. They have been isolated and characterized from Bacillus polymyxa [13], Desulfovibrio gigas [14], Desulfovibrio desulfuricans Norway [15], and Desulfovibrio africanus [16].

2.3. The three-iron ferredoxins

2.3.a. The (3Fe-3S) cluster. Emptage et al. [17], have demonstrated by Mössbauer spectroscopy experiments that the high- and low-potential ferredoxin from Azotobacter vinelandii contains a novel type of cluster. The oxidized low potential center which exhibits an isotropic EPR signal at $g = 2.01$, displays a Mössbauer spectrum compatible with a three-iron cluster. These data were also determined by X-ray crystallography by Stout et al. [18]. Further structural studies have demonstrated the nature of this new cluster to be a (3Fe-3S) center linked to the polypeptide chain by five S of cysteine residues and $L_x$ which is a non-cysteinyl ligand [19,20].

2.3.b. The (3Fe-4S) cluster. The second sign of a three-iron center was found by Huynh et al. [21], in D. gigas ferredoxin II. This protein had originally been reported to be a tetramer of a basic subunit, identical to the subunit of the ferredoxin I which is a trimer [22]. Ferredoxin II has a particular redox potential ($-130$ mV) and EPR signal in oxidized conditions [23]. The Mössbauer spectra, in both oxidation states (3+ and 2+), were indeed almost identical to those from the 3Fe cluster from A. vinelandii ferredoxin [21]. As this ferredoxin contains only one cluster per subunit, D. gigas ferredoxin II has become the standard protein for investigative methods applied to 3Fe clusters.

Although it has been widely assumed that all 3Fe clusters identified by Mössbauer spectroscopy and by the presence of a $g = 2.01$ EPR signal, have the same structure as A. vinelandii ferredoxin I, Beinert et al. [24], suggested, from EXAFS measurements, that the 3Fe cluster of D. gigas ferredoxin II is different. Magnetic circular dichroism and Raman resonance show identical data for D. gigas ferredoxin II and C. pasteurianum ferredoxin damaged by hexacyanoferrate. Chemical analysis of the D. gigas ferredoxin II cluster established an iron-sulfur stoichiometry of 3Fe/4S [25]. These data were supported by the interconversion mechanism which involved Fe atoms but no S atom.

Ferredoxin I isolated from D. gigas is a trimer of the same basic subunit but contains one (4Fe-4S) cluster per subunit. Ferredoxin I and ferredoxin II act as electron carriers in the phosphorolytic reaction and the reduction of sulfate, respectively [22]. It was thought that the different redox centers were stabilized according to the oligomeric state of the protein. The relationship between ferredoxin I and ferredoxin II was then investigated in terms of interconversion. The reconstitution of D. gigas ferredoxin II with apoprotein, Fe and sulfide, under reducing conditions led to (4Fe-4S) cluster protein formation [26], demonstrating that this 3Fe cluster is bound by the same polypeptide chain as the 4Fe cluster.

It was therefore concluded that two 3Fe centers exist, the 3Fe cluster type of A. vinelandii ferredoxin I and the 3Fe cluster type of D. gigas ferredoxin II. A new structure has been suggested for the latter [25]. The removal of a ferrous ion from the corner of a (4Fe-4S) cube leads to the generation of a free thiol side chain, comparable to the model synthetized (Fig. 2). This model is consistent with the Mössbauer spectrum for the 3Fe cluster. In view of the current uncertainty in the structure, the 3Fe clusters are commonly classified in the (3Fe-xS) centers where $x = 3$ or 4. Evidence for (3Fe-xS) clusters ferredoxin in extreme environment organisms has been obtained in the thermophilic bacteria Bacillus steathermophilus [27], Thermus thermophilus [28], Thermo-
sulfobacterium commune [29], and in the acidic bacteria Bacillus acidocaldarius [30], Sulfolobus acidocaldarius [31], Thermoplasma acidophilum [32], also in the methanogenic organism Methanosarcina barkeri [33] and in Mycobacterium smegmatis [34]. It should be noted that most of these organisms are classified in the group of archaebacterium. A physiological meaning of the interconversion of a (3Fe-4S) duster to (4Fe-4S) was suggested in D. gigas to be an electron transfer control mechanism [35]. However, in T. commune ferredoxin [29,36] the presence of 3Fe clusters appears to be strongly correlated to the presence of oxygen during the purification procedure. Thermus thermophilus was reported to contain a 7Fe ferredoxin very resistant to oxidative degradation, but proton NMR studies of samples of di-thionite-reduced T. thermophilus ferredoxin after treatment with excess solid ferricyanide have been interpreted in terms of conversion of the (4Fe-4S)$_{2}^{2+}$ cluster to a (3Fe-xS) cluster [37].

The most important question remaining is: are the 3Fe clusters due to oxidative damage or do they indeed have biological meaning?

### 2.4. The two-iron ferredoxins

This class of proteins is not only found in eukaryotic photosynthetic organisms but (2Fe-2S) ferredoxins are also present in various bacteria. These proteins have a molecular weight of around 14000 which is higher than the 4Fe and 8Fe ferredoxins. They generally contain only one (2Fe-2S) cluster per molecule and give an EPR spectrum in the reduced state (Fe$^{3+}$ Fe$^{2+}$), with a signal around $g = 1.94$. The amino acid sequence of the known (2Fe-2S) ferredoxins is distinct from those of the clostridial type, in particular the four cysteine residues involved in the cluster binding. (2Fe-2S) Ferredoxins have been isolated from halobacteria from the Dead Sea [38-41]. They are very similar to plant or algae ferredoxins, in the visible absorption and circular dichroism spectra. Their redox potential is about $-350$ mV and they act as electron donors in the reduction of nitrite [42].

A paramagnetic protein was isolated from nitrogen-grown cells of C. pasteurianum (43–45), the redox potential ($-300$ mV), and CD and EPR spectra were found to be identical to other (2Fe-2S) ferredoxins.

Electron carriers of the ferredoxin type that participate in multicomponent monooxygenase systems have been extensively investigated. These proteins include adrenodoxin [46] and putidaredoxin [47,48] which are components of steroid hydroxylase and camphor methylene hydroxylase systems. Analogous proteins have been reported to function in the multienzyme systems that dioxygenate the aromatic substrates benzene [49] and pyrazon [50] to cis-dihydriodols.

The (2Fe-2S) ferredoxin$_{Tol}$ characterized in Pseudomonas putida [51] is different due to its redox potential (about $-110$ mV), even if the optical and EPR spectra are similar to other (2Fe-2S) ferredoxins.

(2Fe-2S) ferredoxins have been described as being present in Escherichia coli [52], Clostridium thermoaceticum [53], Pseudomonas aminovorans, Agrobacterium tumefaciens [54] and photosynthetic bacteria [55].

### 3. BIOLOGICAL FUNCTIONS

Ferredoxins function primarily as electron carrier proteins. The need for ferredoxin in a biological reaction is demonstrated by preparing a crude extract of bacterial cells which is then passed through a DEAE-cellulose column at low ionic strength and under anaerobic conditions. Because of its acidic properties, ferredoxin is adsorbed on DEAE-cellulose and then the unadsorbed solution is depleted of ferredoxin. The activity of ferredoxin is analyzed by reconstitution of the electron transfer chain by adding purified ferredoxin to the extract devoid of ferredoxin.

Generally, all the organisms which are producing or consuming hydrogen possess one or more ferredoxins and most of the bacteria not provided with cytochrome contain ferredoxin.

#### 3.1. The eight-iron and four-iron ferredoxins

The reactions that require (4Fe-4S) cluster ferredoxins can be divided into three separate groups: the reactions in which $H_2$ is evolved, the coupled reactions and the reactions in which $H_2$ is the
Table 1
Biological functions of bacterial 8Fe and 4Fe ferredoxins

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Organism</th>
</tr>
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<tbody>
<tr>
<td>Pyruvate</td>
<td>acetyl phosphate</td>
<td>Clostridium</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>CO₂</td>
<td>Clostridium</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>lactate</td>
<td>M. lactilyticus</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>NADPH</td>
<td>Micrococcus</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>NADH</td>
<td>Chromatium</td>
</tr>
<tr>
<td>Sulfite</td>
<td>Sulfide</td>
<td>Desulfovibrio</td>
</tr>
<tr>
<td>N₂</td>
<td>NH₃</td>
<td>Azotobacter</td>
</tr>
<tr>
<td>NO₂</td>
<td>NH₃</td>
<td>Clostridium</td>
</tr>
<tr>
<td>FMN or FAD</td>
<td>FMNH₂ or FADH₂</td>
<td>Micrococcus</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>reduced flavin</td>
<td>Micrococcus</td>
</tr>
</tbody>
</table>

reductant (Table 1). It has been reported that two (4Fe-4S) cluster ferredoxins accept two electrons from pyruvate oxidation or H₂ oxidation; the (4Fe-4S) ferredoxins appear to function as a single electron carrier.

3.1.1. Reactions in which H₂ is evolved. Hydrogen evolution in anaerobic bacteria involves the oxidation of a substrate to produce reduced ferredoxin and a final step where H⁺ acts as a terminal electron acceptor. An example of such reactions is the phosphoroclastic reaction, which has been extensively described in Clostridia and sulfate-reducing bacteria. In this reaction, reduction of ferredoxin is linked to pyruvate oxidation. In Clostridia, the reduced ferredoxin is oxidized by hydrogenase and the electrons are transferred to H⁺ to produce H₂. In Desulfovibrio, it has been demonstrated that the tetrahemic cytochrome c₃ is an obligate intermediate between hydrogenase and ferredoxin [56,57]. This electron transport chain can be used in the reverse direction when coupled to a sulfite reductase [57] or when Desulfovibrio grows autotrophically under a H₂ atmosphere [58].

3.1.2. Coupled reactions. The role of ferredoxin has been demonstrated in the coupling of substrate oxidations with the reduction of NADP⁺, NAD⁺, FMN, FAD, riboflavin, sulfite to sulfide and N₂ to ammonia. Some of these reactions are catalyzed by specific reductases such as flavoproteins [59]. In photosynthetic organisms reduction of ferredoxin is coupled to excitation of chlorophyll by light and to the reduction of nicotinamide adenine dinucleotide [60]. Reduced ferredo-
**Halobacteria** ferredoxins, which are homologous to chloroplast ferredoxins, were found to be a cofactor in the reduction of nitrite as part of a dissimilatory pathway of nitrate [42]. *Halobacterium halobium* ferredoxin was described as being able to serve as a coenzyme of β-ketoacid oxidoreductases (66).

Various (2Fe-2S) ferredoxins very different from the latter were characterized in bacteria. A (2Fe-2S) ferredoxin was isolated from *C. pasteurianum* cells grown under a nitrogen atmosphere [43]. Although its physiological role remains undetermined, its synthesis is known to be associated with the expression of the *nif* genes [67].

A monoxygenase class of (2Fe-2S) ferredoxin has been reported involving the ferredoxin_I Tol purified from *P. putida* which is a component of toluene dioxygenase from this organism [51]. Anaerobic reductive titrations revealed that this ferredoxin accepts electrons from NADH in a reaction that is mediated by a flavoprotein (ferredoxin_I Tol reductase). Reduced ferredoxin_I Tol can transfer electrons to cytochrome c or to a terminal iron sulfur dioxygenase which catalyzes the incorporation of molecular oxygen into toluene or related aromatic substrates. From the same organism the putidaredoxin involved in the camphor methylene hydroxylase system has been isolated [47,48].

The adrenodoxin type (2Fe-2S) ferredoxin is a component of the steroid hydroxylase system. And two (2Fe-2S) ferredoxins have been reported to function in the benzene and pyrazon deoxygenation [49,50].

### 3.3. The three-iron ferredoxins

The physiological interest in the new class of 3Fe ferredoxin has already been mentioned. *D. gigas* ferredoxin II is reported to act in the sulfite reduction reaction as its redox potential is about −110 mV. But the interconversion of (4Fe-4S) to (3Fe-4S) is still an open question. Thauer and Schönheit [68] described iron storage as a possible function for ferredoxin. One of the uncertain mechanisms proposed for Fe release was a ferredoxin degradation. This degradation to the level of apoprotein would necessitate the involvement of a dechelatase which specifically removes Fe or the whole (Fe-S) cluster. The apoprotein might be reused for the synthesis of ferredoxin when exogenous Fe becomes available again. The interconversion of the (4Fe-4S) cluster to (3Fe-4S) has to be related to the ferredoxin degradation involved in the Fe storage reactions. The question now is: does this interconversion come from oxidative damage or dechelatase activity?

### 4. AMINO ACID SEQUENCES OF BACTERIAL FERREDOXINS

Due to their low molecular weight, amino acid sequence determination of ferredoxins has been greatly facilitated. To date, 31 amino acid sequences of bacterial ferredoxins are known. Comparison of these sequences gives some insights into the evolution of these proteins (Fig. 3). The distribution of cysteine residues gives some insights into the evolution of these proteins (Fig. 3). The correlation of this cysteine distribution with the three-dimensional structure of *Peptococcus aerogenes* ferredoxin [69] shows that each cluster is bound to three closely spaced cysteines (Cys-2aa-Cys-2aa-Cys) in one half of the sequence, and a fourth more distant cysteine followed by proline in the second half. The two halves of the molecule are closely homologous and have been suggested as being the result of a gene duplication [70,71]. Ferredoxin I from *D. africanus* [16] has only four cysteines in positions 11, 14, 17 and 51 and thus is the simplest case of a one (4Fe-4S) cluster ferredoxin (Fig. 4).

*D. desulfuricans* Norway ferredoxin I [15] and *D. gigas* ferredoxin [14] each contain six cysteine residues (positions 11, 14, 17, 21, 44 and 51). By analogy with *P. aerogenes* ferredoxin and *D. africanus* ferredoxin I, cysteines 11, 14, 17 and 51 are involved in the binding of the (4Fe-4S) cluster. The loss of the second cluster is associated with the disappearance of the cysteines in positions 41 and 47 in the other ferredoxins. However, the two cysteines in positions 21 and 44 are conserved. The question arises as to whether the ferredoxins with one (4Fe-4S) cluster per subunit derived from a two cluster ferredoxin such as *P. aerogenes* ferredoxin by the substitution of two cysteines or...
the two cluster proteins derived from a one cluster type, as suggested by Tsukihara et al. [72]. However, because of the homology observed between Desulfovibrio and clostridial ferredoxins, it could be postulated that in the course of evolution the second cluster was lost in D. gigas ferredoxin while the two cysteines in positions 21 and 44 remained, whereas they disappeared completely in D. africanus ferredoxin I. D. africanus ferredoxin III sequence [73] is homologous to the other Desulfovibrio ferredoxins but contains seven cysteine residues. One of the cysteine-containing segments shows exactly the same distribution as clostridial ferredoxins for binding a (4Fe-4S) cluster, but the other shows a unique distribution with a cysteine and a proline residue replaced by aspartic and glutamic acid, respectively, and could possibly accommodate a (3Fe-xS) cluster. In an attempt to correlate the amino acid requirements to the stability of a (3Fe-xS) center we have compared different ferredoxins in which this new cluster has been reported (Fig. 5). Some ferredoxins such as those of D. gigas [14], M. barkeri [74] and T. commune [29] exhibiting the same cysteine distribution as the clostridial ferredoxins have been reported as being able to ligand (3Fe-xS) centers. From these observations it could be concluded that it is not possible to predict the nature of the (Fe-S) center in a protein from its sequence. Moreover, it has been shown that the clusters can be interconverted by chemical treatments, by adding iron to the (3Fe-xS) clusters or by oxidizing the (4Fe-4S) cluster. If the apoprotein is prepared, the protein can be reconstituted; limited amounts of iron favor (3Fe-xS) clusters and excess of iron favors the formation of (4Fe-4S) clusters [75]. It should be noted that ferredoxins, which contain only four cysteines such as B. stearothermophilus ferredoxin and D. africanus ferredoxin I, have stable (4Fe-4S) clusters such as the ones of P. aerogenes ferredoxin. But the presence of six cysteine residues in D. gigas ferredoxin allows the
same amino acid chain to accommodate two types of clusters, namely (4Fe-4S) for the trimer (ferredoxin I) and (3Fe-xS) for the tetramer (ferredoxin II) [21,76].

In the cases of _M. barkeri_ and _T. commune_ ferredoxins, the (3Fe-xS) clusters could presumably be produced by oxidative damage as it has been shown by magnetic circular dichroism [59] and Mössbauer spectroscopy [78]. However, some other ferredoxins isolated from _M. smegmatis_ [34], _B. acidocaldarius_ [30], _T. thermophilus_ [28], _S. acidocaldarius_ [31] and _T. acidophilum_ [32] described as containing (4Fe-4S) and (3Fe-xS) centers, show the same unusual cysteine distribution as ferredoxin III from _D. africanus_. Their molecular weight is greater than _D. africanus_ ferredoxin III (from 8000 to 15000) and in Fig. 5 they are shown aligned with an N-terminal part or a C-terminal part missing, respectively. In all these sequences Cys 14 has disappeared and is replaced by Asp, Glu or Val. _D. africanus_ ferredoxin III is the only ferredoxin reported in which proline in position 52 is replaced by a basic residue (glutamine) and it could be postulated that a salt bridge between these two amino acids induces the appropriate folding of the chain following the cysteine residue. The sequence Cys-Val-Glu(Val/Ile)-Cys-Pro-Val-...-Cys is conserved in a large number of iron-sulfur proteins thought to contain 3Fe centers including _A. vinelandii_ and _D. gigas_ ferredoxins. The X-ray structure of _A. vinelandii_ ferredoxin [18] has previously shown that the
glutamic acid of this sequence could coordinate an iron atom of the 3Fe center by a glutamyl-ε-oxygen. Sweeney [20] has also reported a glutamyl coordination consistent with the NMR spectrum of D. gigas ferredoxin II. It can be postulated that an aspartic acid residue or an acidic group in the loop between Cys 11 and Cys 17 (Ferredoxin III numbering) could coordinate such an iron atom in the same manner. However, a later interpretation of the three-dimensional structure indicates a water molecule as a ligand [79]. The lysine residue (position 15) could also act as a ligand. Obviously a clear model cannot easily be proposed as the problem of the number of ligands of the three-iron center is not solved. Only the three-dimensional structure of A. vinelandii ferredoxin is available to show that the interaction between different parts of the polypeptide chain is important for the stabilization of (3Fe-xS) clusters, and so it is difficult to determine the factors involved in the stabilization of the cluster type on the basis of amino acid sequence comparison alone.

The comparison of homologous proteins from thermophilic and mesophilic bacteria, among which ferredoxins have been extensively studied [80], indicates that the higher thermal stability could depend on only a few differences in their sequences. In some cases, it has been reported that

![Fig. 6. Comparisons of ferredoxins from Clostridium thermocellum [83], Clostridium tartarivorur [84], Clostridium thermosaccharolyticum [115] and Peptococcus aerogenes [116]. Residues common to all proteins are enclosed in boxes and (-) refers to a gap to make all alignments the most probable. The numbers refer to C. thermocellum ferredoxin.](image-url)
enzyme thermostability can be enhanced by single amino acid substitutions [81,82]. The thermal stability of ferredoxins is observed by measuring changes of their absorption spectrum and biological activity upon heat treatment. In the thermostable proteins, bleaching does not occur up to 80 °C and 40% decolorization is obtained after 1 h treatment. Compared to *C. pasteurianum* ferredoxin which is inactivated after 1 h treatment at 70 °C, the physiological activity of thermostable ferredoxins is not lowered. Upon transition from mesophilic to thermophilic, the increase in hydrophobicity by substitution of a few suitable amino acids at the surface of the protein is observed together with the formation of internal ion pairs or hydrogen bonds involving charged and polar residues. The clusters themselves and their immediate surroundings are generally left unchanged and thus do not contribute directly to the thermal stability.

Fig. 6 compares the sequences of ferredoxins isolated from thermophilic bacteria with the ferredoxin isolated from the mesophilic bacterium *P. aerogenes*, its crystallographic structure being known. The differences between the *P. aerogenes* and the *Clostridium thermocellum* ferredoxins are two insertions in positions 22 and 27, and twenty-eight substitutions [83]. It should be emphasized that the effects of basic amino acids in some thermophilic ferredoxins [84] cannot be involved in the heat stability, since *C. thermocellum* ferredoxin lacks the histidine residues in positions 2 and 24 and lysines in positions 29 and 53. Structural considerations for the high thermal stability of *C. thermocellum* ferredoxin have been proposed on the basis of a model of the three-dimensional structure derived from the *P. aerogenes* crystallographic structure. This model is based on sequence alignment, display handling and energy minimization calculations [83]. According to this study, the thermostability should not involve special interactions with the clusters, but some hydrophobic substitutions on the surface of the molecule together with the formation of specific salt bridges between the N and C terminal ends. In *P. aerogenes* (Fig. 7) the positively charged N terminal is surrounded by four negative charges, leading to a predominant 1–37 interaction and some weak secondary effects. In *C. thermocellum*, two positively charged groups are balanced by two negative charges, allowing the formation of specific salt bridges. This striking effect could firmly lock the N and C terminal ends together with the loop bearing residue 37. This hypothesis must be confirmed by the three-dimensional structure of *C. thermocellum* ferredoxin which is being elucidated at present.

5. PHYLOGENETIC CONSIDERATIONS OF FERREDOXIN SEQUENCES

Because bacterial ferredoxins are found in a broad spectrum of organisms and because their
small molecular size facilitates their structural study, the molecular evolution of ferredoxins has been extensively studied and detailed phylogenetic trees have been proposed [85].

Thirty-one bacterial type ferredoxins have been aligned in Fig. 3. A gene doubling is shared by all the sequences and has been proposed as having occurred early in the course of evolution [70,71,86]. A reconstitution experiment using half molecules of *C. acidi-urici* ferredoxin [87] suggests that a primordial ferredoxin of about 30 amino acids was synthesized with iron and sulfur under the conditions that existed on the primitive Earth. The ancestral form of ferredoxin must have been internally duplicated to produce an active electron carrier similar to the present day ferredoxin. The most parsimonious phylogenetic tree proposed by Fitch and Bruschi [88] (Fig. 8) shows that the 31 sequences are divided into six major groups (from top to bottom).

*Pseudomonas* and *Azotobacter* (5 species)
*Clostridia* (7 species)
Photosynthetic bacteria (5 species)
Eubacterial thermophiles (5 species)
*Desulfovibrio* (4 species)
Archaeabacteria (5 species)

The tree shows reasonable relationships in that photosynthetic, thermophilic and *Desulfovibrio* organisms are grouped together. However, some anomalies exist. The exception to the coherence of the thermophiles is *T. thermophilus* which is placed close to *B. acidocaldarius* and *Pseudomonas*. Another exception is *Rhodospirillum rubrum* which is more similar to *C. pasteurianum* than is any other *Clostridium*. Comparison of archaeabacterial ferredoxin sequences with the eubacterial or eukaryotic ones does not show a ferredoxin phylogenetic line proper to the archaeabacteria. This does not seem to agree with the proposal [89] that archaeabacteria, defined as a group of bacteria living in extreme environments, constitute a very ancient kingdom distinct from eubacteria and eukaryotes. On the basis of ferredoxin sequences, some authors have suggested that they could not be classified into one group in terms of bacterial phylogeny but that they have probably multiple origins and were derived from other bacteria [31,90]. In contrast to the phylogenetic tree proposed by Fox et al. [91], based on 16S rRNA comparisons, methanobacterial ferredoxins are placed together with the halobacterial. In the tree (Fig. 8), among all the sequences, *Methanosarcina*
ferredoxin is the most similar to the Desulfovibrio. The relative place of Chromatium, Rhodopseudomonas and Desulfovibrio is not the same in these two different trees.

The presence of a common ancestral form between plant and bacterial ferredoxins has been suggested by Matsubara et al. [92], Keresztes-Nagy et al. [93] and Schwartz and Dayhoff [94]. In spite of the differences in their chromophore structures, a similarity is recognized in the primary structures and a phylogenetic tree has been constructed to organize all bacterial and chloroplast-type ferredoxins [95]. In this model, the (2Fe-2S) ferredoxins from the chloroplast type and the halobacterial type have been generated by gene tripllication of the ancestor followed by structural change of the cluster.

A comparison of the sequence of the (2Fe-2S) ferredoxin isolated from C. pasteurianum [96] failed to detect any homology with the other ferredoxin sequences containing 2Fe, 3Fe and 4Fe active sites. In particular, the cysteine spacing is different in the C. pasteurianum (2Fe-2S) ferredoxin and in (2Fe-2S) ferredoxins of the chloroplast type. Thus, it appears that the stabilization of (2Fe-2S) clusters in ferredoxins is not achieved in a unique and characteristic pattern of cysteine ligands, and several solutions have been found in the course of evolution.

To establish phylogenetic comparisons among large groups of organisms, ferredoxin sequences are extremely useful, together with other proteins, nucleic acid sequences [85] and 16S rRNA sequences [91,97]. For example, Dayhoff has proposed a comprehensive evolutionary tree [85] derived by superimposing trees from sequences of c type cytochromes, ferredoxins and SS rRNA. Several lines diverge near the base of the tree, i.e., Clostridium, Megasphaera and Peptococcus, which are all anaerobic heterotrophic bacteria. Two other lines lead to Chromatium and Chlorobium which are anaerobic bacteria capable of photosynthesis using H₂ as an exogenous electron donor.

Then, another line leads to the ‘late bacterial ancestor’, the common ancestor of B. stearothermophilus, Desulfovibrio, Pseudomonas and M. smegmatis. Sulfate-reducing bacteria respire anaerobically using sulfate as the terminal acceptor, whereas B. stearothermophilus is a facultative aerobe; Pseudomonas and M. smegmatis are aerobic bacteria.

Recent progress in sequence studies on nucleic acids has broadened the field of molecular evolution, allowing combined comparisons with protein structural data to study the mechanisms of biological evolution. Three-dimensional structural studies have also been introduced by Dickerson [98] to investigate the molecular evolution of cytochrome c; it is not questionable that, in the same way, the elucidation of the three-dimensional structure of different ferredoxins will facilitate the establishment of phylogenetic relationships between them.

6. CRYSTALLOGRAPHIC STUDIES

Because of the lability of its chromophores to oxygen, ferredoxin can be difficult to crystallize. Only three ferredoxin structures provide a clear image of the three (Fe-S) clusters known at present [99]. Spirulina platensis ferredoxin [100] contains a typical (2Fe-2S) cluster, A. vinelandii ferredoxin [79] illustrates the (3Fe-3S) core structure and P. aerogenes ferredoxin [69] is representative of the (4Fe-4S) clusters. However, various bacterial ferredoxins have been crystallized (Table 3) at high ionic strength and, except Halobacterium (2Fe-2S) ferredoxin, the salt used has been (NH₄)₂SO₄. The lability of labile sulfur presents an additional problem in the preparation of heavy derivatives.

The structure of S. platensis ferredoxin gives a good picture of the (2Fe-2S) center in close agreement with the analog compound structures [99]. As Halobacterium (2Fe-2S) ferredoxins are very similar to S. platensis ferredoxin in primary structure, optical characteristics and electron paramagnetic resonance spectrum [101], the distances and angles for the (2Fe-2S) cluster given in Table 4 can be extended to Halobacterium ferredoxins. On the other hand, the (2Fe-2S) ferredoxin isolated from C. pasteurianum is so different from S. platensis in the amino acid sequences [96] that new parameters must be considered for the (2Fe-2S) cluster of C. pasteurianum ferredoxin.
Table 3
Ferredoxin crystallization data

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Bacterium</th>
<th>Crystal data</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2Fe-2S)</td>
<td>Halobacterium</td>
<td>Hexagonal crystal form space group $P_6_322$</td>
<td>[101]</td>
</tr>
<tr>
<td>(3Fe-xS)</td>
<td>Azotobacter vinelandii</td>
<td>Tetragonal crystal form space group $P_4_2_1_2$</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td>Desulfovibrio gigas</td>
<td>(1) orthorhombic crystal form space group $P_2_1_2_2$</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td>(2) monoclinic crystal form space group $C_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4Fe-4S)</td>
<td>Bacillus thermoproteolyticus</td>
<td>Plates form crystal $P_1$</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>Clostridium</td>
<td>Rectangular prisms</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. acidi-urici</td>
<td>Rosettes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. tetanomorphum</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. pasteurianum</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. butyricum</td>
<td>Microcrystals</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. cylindrosporum</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. thermocellulium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peptococcus aerogenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Desulfuricans Norway</td>
<td>Octahedra crystal form space group $P_4_3_2$</td>
<td>[120]</td>
</tr>
<tr>
<td>(4Fe-4S)</td>
<td>Chromatium vinosum</td>
<td>Orthonohedral crystal form space group $P_2_1_2_2$</td>
<td>[103,104]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4
Comparative interatomic distances and angles in (2Fe-2S), (3Fe-3S), (4Fe-4S) and HIPIP clusters

<table>
<thead>
<tr>
<th>Cluster</th>
<th>2Fe-2S</th>
<th>3Fe-3S</th>
<th>4Fe-4S</th>
<th>HIPIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-Fe</td>
<td>2.72 Å</td>
<td>4 Å</td>
<td>2.73 Å</td>
<td>2.72 Å</td>
</tr>
<tr>
<td>$S_1S_1$</td>
<td>2.94 Å</td>
<td>4.05 Å</td>
<td>3.52 Å</td>
<td>3.55 Å</td>
</tr>
<tr>
<td>Fe-$S_1$</td>
<td>2.0 Å</td>
<td>2.34 Å</td>
<td>2.23 Å</td>
<td>2.26 Å</td>
</tr>
<tr>
<td>Fe-$S$</td>
<td>2.1 to 3.0 Å</td>
<td>2.28 Å</td>
<td>2.22 Å</td>
<td>2.20 Å</td>
</tr>
<tr>
<td>Fe-$S$-Fe</td>
<td>85°</td>
<td>117°</td>
<td>75°</td>
<td>74°</td>
</tr>
<tr>
<td>$S_1$-$Fe$</td>
<td>100°</td>
<td>119°</td>
<td>115°</td>
<td>115°</td>
</tr>
</tbody>
</table>

To date, the (3Fe-3S) cluster geometry has been revealed by crystallographic techniques only in A. vinelandii ferredoxin [79]. The geometry data (Fig. 9) show that although the (3Fe-3S) cluster displays essentially standard (Fe-S) bond lengths, the Fe-Fe distances are 1.3 Å greater than in (2Fe-2S) and (4Fe-4S) clusters. The implication of this arrangement is that there is little direct interaction of Fe orbitals. The average $S_1$-$S_2$ distance is only 0.5 Å larger than for (4Fe-4S) clusters. By comparing the internal angles of the core, a marked difference is seen with the (3Fe-3S) cluster where they are essentially 120°. The angles at labile S atoms are enlarged by 40° from the values observed in (2Fe-2S) and (4Fe-4S) clusters.

From the cubane (3Fe-4S) cluster, only crystallization and a preliminary X-ray diffraction study of D. gigas ferredoxin II has been reported [102]. The Patterson map is consistent with a (3Fe) cluster but, from the Fe-Fe distances (2.8 Å and 3.4 Å), it is not yet possible to establish the nature of the (Fe-S) cluster conformation.

Four independent structures of (4Fe-4S) clusters have been determined from the protein crystal structures of Chromatium vinosum HIPIP in oxidized and in reduced forms [103,104], and of P. aerogenes [69] (Fig. 10) and A. vinelandii [79] (Fig. 9) ferredoxins. From these structures it appears that the (4Fe-4S) clusters are essentially the same. The interatomic distances for HIPIP and P. aerogenes ferredoxin are summarized in Table 4. Nevertheless, subtle but significant differences in the stereochemistry of the HIPIP, in oxidized and reduced states, and oxidized ferredoxin have been observed and correlated with the reduction potentials.

7. CONCLUSIONS

Recent compilations of data on bacterial (Fe-S) proteins show the continuing interest in the area. During the last few years evidence of the new (3Fe-xS) clusters was a new means of iron-sulfur
proteins study, and this helped to readjust views and ideas. This characterization of a new cluster also demonstrates the ambiguity in the analysis of iron-sulfur-containing proteins. In this way, *A. vinelandii* ferredoxin I was first described as a (2Fe-2S) and (4Fe-4S) ferredoxin [105]. The evidence of the (3Fe-3S) cluster was suggested by complementarity of Mössbauer, EPR and X-ray studies. Finally, one of the problems in this field is the finding of a suitable methodology for the determination of the cluster type of iron-sulfur proteins [106].

The interconversion of 3Fe and 4Fe clusters without any auxiliary factors or enzyme interaction shows how fragile and malleable iron-sulfur proteins are. In this field, new lines of thinking are taking place. The current status of research on ferredoxin leaves a number of important questions which need to be answered. In particular, the biological significance of the new (3Fe-xS) cluster and, more generally, the mechanism by which the (Fe-S) cluster is inserted into the polypeptide chain in the cell.

The chemical reconstitution has been exten-
Fig. 10. X-ray crystal structure of Peptococcus aerogenes ferredoxin [from 69].

eissively studied [11,12] and enzymatic synthesis has been obtained by using a sulfur transferase in the presence of thiosulfate and DL-dihydrolipoate [107,108]. The enzymic synthesis and chemical reconstitution allow two primary conclusions on C. pasteurianum 8Fe-ferredoxin folding: the nature of the cluster is determined by the apoprotein itself and the refolding of the protein is the rate-limiting step of the reconstitution. Considering that only a few bacterial species have been shown to contain the rhodanese activity, one of the controversial possibilities is the presence of other sulfur-transferases or sulfide insertase in these organisms, but that remains to be demonstrated [109]. Takahashi et al. [110] described a physiological pathway by which the iron-sulfur cluster is introduced into ferredoxin using cysteine as a sulfide donor in intact chloroplasts.

Graves et al. [111–112] who were interested in examining the individual steps involved in the conversion process of apoprotein to active ferredoxin, have undertaken the cloning and the study of the in vivo and in vitro transcription of the C. pasteurianum ferredoxin gene. These studies may also provide insight into the basis for the high level of ferredoxin expression in Clostridia. Analysis of the C. pasteurianum genomic DNA indicates that the ferredoxin gene is present in a single copy. According to the DNA sequence, the only post-translational processing of this small apoprotein is the hydrolysis of the initiator methionine. Regions homologous to typical E. coli and vegetative Bacillus subtilis promoters and terminators are found near the ferredoxin coding region. E. coli RNA polymerase most likely recognizes the same promoter (P₁) as the clostridial polymerase and, furthermore, efficiently uses an additional promoter (P₂) that is poorly recognized by the normal host enzyme. Comparisons of the ferredoxin promoter with Gram-positive promoters reveal some conserved sequences, indicating the presence of the 'extended' promoter recognition site in Gram-positive organisms.

Recently [113], the expression of Desulfovibrio vulgaris Hildenborough hydrogenase in E. coli has been reported. The two subunits (46 kDa and 13.5 kDa) are expressed in E. coli but in an enzymatically inactive form. The active hydrogenase from D. vulgaris H. and the inactive derived hydrogenase from E. coli have been compared in parallel EPR measurements, under a variety of redox conditions. The data obtained indicate that E. coli provides the environmental conditions that are sufficient for the synthesis and assembly of two regular ferredoxin-like cubanes. However, the third (Fe-S) cluster, the active site, is not assembled at all. It should also be noted that the intactness of the active site is not a prerequisite to obtain the native conformation of the protein and it could be concluded that it is not the (Fe-S) cluster that determines the protein conformation.

The recent development of the genetic approach on iron-sulfur proteins points to ferredoxin as a simple model for the study of the biosynthesis of an apoprotein and its conversion to a functional (Fe-S) cluster-containing form.
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