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STRUCTURAL AND FUNCTIONAL DIVERSITY OF FERREDOXINS AND RELATED PROTEINS

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I. Introduction

The term "ferredoxin" was coined by D. C. Wharton (*1*) and applied to a nonheme iron protein isolated from nonphotosynthetic anaerobic bacteria, *Clostridium pasteurianum* (*2, 3*). The protein served as an

electron-transferring factor in the phosphoroclastic reaction of pyruvate oxidation linked with nitrogen fixation in this organism. Immediately afterward, another protein isolated from spinach chloroplasts was similarly named ferredoxin on the basis of the exchangeability of the proteins from different organisms in supporting photoreduction of $NADP^+$ by spinach chloroplasts (*4*). The presence of inorganic sulfides in these ferredoxins was recognized (*5, 6*), and these two classes of ferredoxins became the first representatives of iron–sulfur (Fe–S) proteins. However, as further studies have progressed, other ferredoxins of different types and related proteins similar to ferredoxins have been recognized (*7–10*). There are now five classes of ferredoxins defined in terms of their Fe–S cluster(s): [2Fe–2S], [4Fe–4S], [3Fe–4S], 2[4Fe–4S], and [4Fe–4S][3Fe–4S]. In each class, however, we see further diverse structures and functions of these proteins, which have been isolated from organisms ranging from higher plants and animals to diverse microorganisms. For some, only the gene structures have been elucidated, and as a consequence of recent rapid progress in gene isolation and sequencing, the functions of their putative proteins can only be speculated. The present review will focus on ferredoxins and related proteins that have been relatively recently reported and that are of interest to the authors. In particular, the diverse structural and functional features of these proteins in one organism are emphasized. For earlier work readers should consult review articles that have appeared previously (*7–14*).

II. [2Fe–2S] Ferredoxins and Related Proteins

A. Ferredoxins in Oxygenic Photosynthetic Organisms

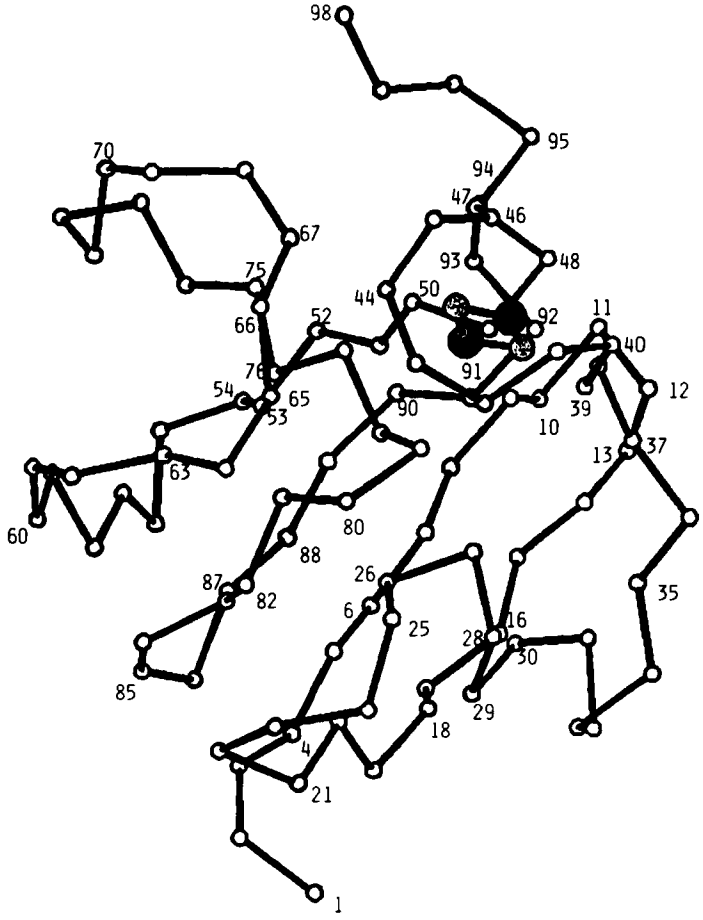
Many, but not necessarily all, oxygenic photosynthetic plants and algae, including cyanobacteria (or blue-green algae), contain at least two molecular species of [2Fe–2S] ferredoxin (*11–15*). These iso-forms function as electron carriers in diverse metabolic pathways, such as the photosynthetic electron transfer chain, nitrite reduction, sulfite reduction, glutamate synthesis, nitrogen fixation, thioredoxin oxidoreduction, and lipid desaturation (*16–19*), but the distinction between the iso-forms on the basis of their functions has been unclear (*20*), except for the heterocyst ferredoxin of the nitrogen-fixing cyanobacterium, *Anabaena* (*21–25*). In general, [2Fe–2S] ferredoxins in plants and algae are very acidic (p*I* values from 3 to 4). They have absorption maxima in the visible region at about 330, 420, and 465 nm in the oxidized state,

with some slight variations depending upon source. On reduction, the absorbances decrease about 50% with no defined absorption maximum. The reduction potentials of these ferredoxins are very low, centered at around −400 mV.

So far, we have complete amino acid sequences of over 75 ferredoxins isolated from land plants (*26–50*), eukaryotic algae (*51–60*), and prokaryotic algae (*61–85*), including those deduced from DNA sequences in several cases (*29, 40, 42–44, 70, 72–74, 79, 84, 85*). The number of amino acid residues ranges from 93 to 99, giving a molecular mass of about 11 kDa, and they are all homologous, indicating that the molecules have been well conserved over 1.5 billion years, particularly the residues around the [2Fe–2S] cluster (*11, 86*).

Three-dimensional structures of three cyanobacterial ferredoxins are now known (*87–89*) and show essentially the same polypeptide chain folding motif (Fig. 1). When their sequences are aligned they are representative of two types of this class of ferredoxin (*12*). The *Spirulina* and *Aphanothece* ferredoxin molecules have a β-barrel-like structure and the [2Fe–2S] cluster is held in the polypeptide chain through four Fe–S bonds. Three of these Fe–S bonds are in a loop segment from positions 40 to 49 (*Spirulina* ferredoxin numbering). A hydrophobic core with several conserved residues is present inside the β-barrel-like structure. One of the two iron atoms should be more easily reduced than the other, indicated by the differences in the hydrogen bonding and hydrophobicity around the iron atoms (*87, 88*). A horsetail (*Equisetum arvens*) ferredoxin I (*46*) has recently been crystallized and analyzed at 1.8 Å, indicating the presence of small helical structures near positions 65–70 and the C-terminal portion (T. Tsukihara, personal communication, 1991) in addition to those found in the previous analyses (*86–88*). A crystallographic analysis of *Anabaena* PCC7120 ferredoxin has recently shown some secondary structural differences (*89*) compared to those previously reported for *Spirulina* ferredoxin (*87*). The refined analysis revealed four strands of β-pleated sheet and three α-helical segments.

The amino acid sequence of one of the two ferredoxins from *Aphanothece sacrum* (a unicellular cyanobacterium), ferredoxin I (*82*), has two gaps at positions 10 and 14 in the alignment given in Fig. 1. Ferredoxins of this type include those of all land plants, exemplified by spinach ferredoxin I (*27*) (Fig. 2a), except for ferredoxins of some tissues or organs as described below, and most of the eukaryotic algae (*12*); a red alga, *Rhodymenia palmata* (*59*), in the family Florideophyceae; and a unicellular cyanobacterium, *Synechocystis* 6714 (*80*). The sequence of *Spirulina platensis* (a filamentous cyanobacterium) ferredoxin (*63*) has

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   1          .          .          .          .
(a) ATYKVTLINEAEGINETIDCDDDTYILDAAEEAGLDLPYSCRAGACSTC
(b) ASYKVTLKT-PDG-DNVITVPDDEYILDVAEEEGLDLPYSCRAGACSTC
    .          .          .          .          .     98
(a) AGTITSGTIDQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIKTHQEEGLY
(b) AGKLVSGPAPDEDQSFLDDDQIQAGYILTCVAYPTGDCVIETHKEEALY
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FIG. 1. Schematic representation of the main chain folding of *Spirulina platensis* [2Fe–2S] ferredoxin. The protein is shown as a stick and ball model based on Ref. *87* as modified by the CRC macromolecular display program (courtesy of Dr. K. Fukuyama). Large filled and stippled circles represent iron and sulfur atoms of the Fe–S cluster, respectively. The amino acid sequences of *S. platensis* ferredoxin (a) (*63*) and *Aphanothece sacrum* ferredoxin I (b) (*82*) are given for two representative ferredoxins. Amino acids are shown by one-letter abbreviations (IUPAC–IUB–JCBN). Italicized *C* is the cysteine residue chelating the iron atom. Gaps are inserted to align the sequences for highest homology.

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(a) -AA-YKVTLVT-PTG-NVEFQCPDDVYILDAAEEEGIDLPYSCRAGSCSSC
(b) -SAVYKVKLIG-PDGQENEFDVPDDQYILDAAEEAGVDLPYSCRAGACSSC
(c) -AT-YKVTLITKESG-TETFDCPDDVYVLDQAEEEGIDLPYSCRAGSCSSC
(d) -AT-YKVTLINEEEGINAOLEVADDQTILDAGEEAGLDLPSSCRAGSCSTC
(e) -AT-FKVTLINEAEGTKHEIEVPDDEYILDAAEEQGYDLPFSCRAGACSTC
(f) -AS-YQVRLINKKQDIDTTIEIDEETTILDGAEENGIELPFSCHSGSCSSC
(g) -AT-YQVEVIY--QGQSQTFTADSDQSVLDSAQAAGVDLPASCLTGVCTTC
(h).DDLFEKAADAGLDGEDYGTMEVAEGEYILEAAEAQGYDWPFSCRAGACANC
(i) ---MDKATLTF--TDVSITVNVPTGTRIIEMSEKVGSGITYGCREGECGTC
(j)..GLFVPPPESTVSVRGQGFQFVPRGQTILESALHQGIAFPHDCKVGSCGTC

(a) AGKLKTGSL-NQDDQSFLDDDQIDE-----GWV-LTCAAYPVSDVTIETHKEEELTA
(b) AGKIEKGQV-DQSEGSFLEDHHFEK-----GYV-LTCVAYPQSDLVIHTHKEEELF
(c) AGKVVAGSV-DQSDQSFLDDDQIEA-----GWV-LTCAAYPSADVTIETHKEEELTA
(d) AGKLVSGAAPNQDDQAFLDDDQLAA-----GWV-MTCVAYPTGDCTIMTHQESEVL
(e) AGKLVSGTV-DQSDQSFLDDDQIEA-----GYV-LTCVAYPTSDVVIQTHKEEDLY
(f) VGKVVEGEV-DQSDQIFLDDEQMGK-----GFA-LLCVTYPRSNCTIKTHQEPYLA
(g) AARILSGEV-DQPDAMGVGPEPAKQ-----GYT-LLCVAYPRSDLKIETHKEDELYALQFGQPG
(h) ASIVKEGEI-DMDMQQILSDEEVEE-----KDVRLTCIGSPAADEVKIVYNAKHLDYLQN
(i) MTHILEGSE-NLSEPTALEMRVLEENLGGKDDR-LACQCRVLGGAVKVRPA
(j) KYKLISGRV-NELTSSAM---GLSGDLYQSGYR-LGCQCIPKEDLEIELDTVLGQALVPIET..
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FIG. 2. Amino acid sequences of [2Fe–2S] ferredoxins and ferredoxin-like proteins. Gaps are inserted to align the sequences for maximum homology. Italics show the residues emphasized in the text. (a) Spinach ferredoxin I (*27*), (b) radish root ferredoxin B-1 (*41*), (c) *Silene platensis* ferredoxin (*40*), (d) *Aphanothece sacrum* ferredoxin II (*83*), (e) *Anabaena* PCC7120 ferredoxin I (*70*), (f) *Anabaena* PCC7120 heterocyst ferredoxin (*73*), (g) *Synechococcus* 6301 putative ferredoxin (*79*), (h) *Halobacterium halobium* ferredoxin (*135*) (the N-terminal 20 residues are omitted), (i) *Rhodobacter capsulatus* ferredoxin-like protein (*139*), and (j) *Pseudomonas* XylA protein (*161*), the sequence of which starts from residue 5 and lacks the C-terminal portion.

no gap at these positions, as also is true of *A. sacrum* ferredoxin II (*83*) (Fig. 2d) as well as the ferredoxins of many other cyanobacteria, including heterocystous cyanobacteria (*73*); red algae in the Protoflorideophyceae; and the yellow-green alga, *Bumilleriopsis filiformis* (*12, 58*). An intermediate form is seen in the ferredoxins of a marine, unicellular green alga, *Bryopsis maxima* (*51*); three cyanobacteria, *Synechococcus* sp. (*76*), *Aphanizomenon flos-aquae* (*67*), and *Nostoc* MAC I (*69*); and higher plant nonphotosynthetic tissues (*41, 42*); these have only one gap at position 12 in the alignment given in Fig. 2, as exemplified by radish root ferredoxin I (*41*) (Fig. 2b). Another intermediate form is found in *Silene platensis* ferredoxin (*40*) with the gap at position 16 (Fig. 2c). These two positions are located near the corner of the first turn from the N terminus of the polypeptide chain (Fig. 1). Ferredoxins with deletions at both of the two positions always have a proline residue at position 13 (*12*), which emphasizes the turn at the corner (*88*). These

two events, the deletions and introduction of proline, probably occurred in concert during evolution (*50*). *Aphanothece sacrum* has two representative ferredoxins, I and II; the evolutionary implication of this has been discussed elsewhere (*50, 83*).

Many organisms have iso-forms of ferredoxin; the comparisons of the sequences in phylogenetic trees have revealed the relationships among ferredoxins (*11, 12, 56, 90–92*) and have led to the interpretation that gene duplications occurred long before speciations (*11, 12, 46, 83, 92*). Further, it has been suggested that [2Fe–2S] ferredoxins were derived from an ancestral form of clostridial-type 2[4Fe–4S] ferredoxin through unequal crossing-over of genes, differentiation, and translocation (*93*). Arguments for a common origin for the two different types (*27, 31, 90, 94, 95*) and counterarguments (see Ref. *8* and references therein) have been given.

The question as to whether the iso-forms of ferredoxin have different functions has not yet been answered by comparisons using various enzymatic assay systems (*20, 96, 97*), although amino acid compositions and reduction potentials of the iso-forms can be significantly different (*13, 14, 69, 98–100*). However, a distinct functional difference was found for the ferredoxins of vegetative cells and heterocysts of a nitrogen-fixing filamentous cyanobacterium, *Anabaena variabilis* (*21–24*).

Most cyanobacteria that fix nitrogen aerobically do so in specialized cells called heterocysts, which develop when the organism is grown in a medium deficient in nitrogen sources (*101*). Reduced heterocyst ferredoxin donated electrons to both *A. variabilis* nitrogenase and the $NADP^+$ photoreduction system, but the vegetative cell ferredoxin was almost inactive in the nitrogenase system, suggesting a unique protein conformation of the heterocyst ferredoxin enabling its interaction with nitrogenase (*21*). The two ferredoxins showed some differences in their molecular masses, visible absorption spectra, p*I* values, and electron paramagnetic resonance (EPR) spectra, but their amino acid compositions, immunological properties, and midpoint reduction potentials were significantly different (*23*). Heterocyst ferredoxin had a reduction potential of -405 mV and vegetative cell ferredoxin had a reduction potential of -433 mV. Heterocysts contained another ferredoxin as a minor component; this was identical to vegetative cell ferredoxin and its function was assumed to be in cyclic photophosphorylation, supplying energy for nitrogen fixation (*23, 73*).

A related heterocystous cyanobacterium, *Anabaena sphaeria,* was shown to have two ferredoxins with biochemical properties and amino acid compositions that were nearly identical. They differed in molecular mass, 33,600 Da for ferredoxin I and 11,500 Da for ferredoxin II, and

only the latter was an effective electron donor to nitrogenase and hydrogenase (*25*).

Recently, the structural gene, *fdxH*, for *Anabaena* PCC7120 heterocyst ferredoxin was isolated and sequenced to deduce the encoded amino acid sequence (*73*) shown in Fig. 2f; for comparison, the amino acid sequence of the vegetative cell ferredoxin is shown in Fig. 2e (*70*). Both have 98 residues, which is typical for cyanobacterial ferredoxins (*12*), but 47 out of 98 residues in the aligned sequences are different, with a lesser number of acidic residues in heterocyst ferredoxin giving a higher p*I* value, 3.0, than that (below 2.7) of the vegetative ferredoxin (*23*). Although only a partial sequence of the N-terminal 40 residues of another heterocyst ferredoxin of *Anabaena* ATCC29413 is available, it is apparent that the number of amino acid differences between heterocyst ferreodoxins in different strains of *Anabaena* is much smaller than that between heterocyst ferredoxin and vegetative cell ferredoxin in the same strain (*73*). This phenomenon is the same as those found for ferredoxin iso-forms in other cyanobacteria (*11, 12, 92*), but in particular the heterocyst ferredoxins seem to be developed to function specifically in nitrogen fixation. The alterations in amino acids around the Fe–S cluster, a loop region, from Arg 44 of vegetative ferredoxin (in the alignment of Fig. 2) to His of heterocyst ferredoxin, from Ala 45 and 47 to Ser, and from Thr 50 to Ser, might be responsible for the change of reduction potential and for critical interaction with nitrogenase (*73*). However, in contrast with similar changes at the loop area found between *Nostoc* strain MAC ferredoxins I and II, the reduction potential of a heterocyst ferredoxin appears to be higher than that of the vegetative ferredoxin.

Heterocyst ferredoxin is less acidic than vegetative ferredoxin and the distribution of the sites giving this charge difference was examined using the three-dimensional structural model of *Spirulena platensis* ferredoxin (*73*). They are positions 68, 12–15, 17–19, 23, 26, 57, 59, 75, 76, 87, 89, 93, 98, and 99. One notable difference was Ser in the vegetative cell ferredoxin and Ile in the heterocyst ferredoxin, at position 67, which is located near the cluster, where other ferredoxins except a putative second ferredoxin from *Synechococcus* (described later) have Ala, Thr, Ser, Asn, Gln, or Ala (Fig. 2). This region was suggested to interact with ferredoxin-$NADP^+$ reductase (*102, 103*). The hydrophobic nature of this area may also be necessary to interact with nitrogenase. However, it is improbable that exchange of comparatively few amino acids would produce the large functional difference of ferredoxins derived by trial and error during evolution; therefore, elucidation of the structure–function relationship of the nitrogenase-specific ferredoxin

must await availability of the three-dimensional structure of the heterocyst ferredoxin. Site-directed mutagenesis experiments may also be useful to study the relationship (*104*).

Two iso-forms (I and II) of ferredoxin exist in mature spinach leaves (*105*) as well as in etiolated cotyledons (*30*). The two spinach ferredoxin sequences have 25 amino acid differences out of 97 residues, reflecting microenvironmental difference at the Fe–S clusters (*106, 107*).

A ferredoxin in bean sprouts (*108*) proved to have properties similar to those of spinach ferredoxin. Another ferredoxin was found in bean stems (*109*). The ratio of these ferredoxins varied during seedling development (*30*), with the relative content of ferredoxin II compared to ferredoxin I being higher in spinach etiolated tissues. Synthesis of ferredoxin II occurred in contrast with ferredoxin I was light independent. A multifunctional electron carrier encoded by the ferredoxin II gene was present before gene duplication, and was involved with nonphotosynthetic electron pathways such as nitrite reduction, glutamate synthesis, etc., in early evolutionary time. The second ferredoxin, I, was produced by gene duplication with functions equivalent to ferredoxin II. These two ferredoxins subsequently became differentiated by accumulating mutations in association with the development of complicated metabolic systems, with the expression of the ferredoxin genes becoming divergent (*30*). The different expressions of the genes for ferredoxin iso-forms have been observed under variable growth conditions (*13, 20, 110, 111*). With this likelihood of distinct functions of ferredoxin isoforms, the tissue- or organ-specific expression of ferredoxin genes in higher plants has become a target of recent research (*41, 42, 96, 112–115*).

Maize, a C_4 plant that evolved relatively recently, has a CO_2 fixation pathway modified from C_3 plants such as spinach. In maize, cell differentiation has occurred such that the bundle sheath cells have chloroplast thylakoids that differ morphologically from those of C_3 plants, whereas mesophyll cells retain thylakoids similar to those of C_3 plants but have a changed chloroplast enzymology (*116*). Four ferredoxin isoforms [and possibly one more (*42*)] were isolated from maize seedlings. All four iso-forms, I–IV, were present in green and etiolated leaves, but only ferredoxins III and IV were detected in nonphotosynthetic organs such as mesocotyl and root. Ferredoxins I and II, the leaf-specific iso-forms, were light dependent, suggesting that they were involved in photosynthesis, but the non-organ-specific ferredoxins III and IV were light independent (*115*). Ferredoxin I was found in mesophyll and bundle sheath cells, but ferredoxin II was found only in bundle sheath cells. Although distinct functions of these four ferredoxins are still

obscure, the occurrence of a specific iso-form of ferredoxin, ferredoxin II in this case, in bundle sheath chloroplasts suggested this was adapted to a specific metabolic role in these chloroplasts (*115*).

Three maize ferredoxin cDNAs were cloned and sequenced to deduce the amino acid sequences of these iso-forms (*42*). The transit peptide of ferredoxin III was different from those of the other two leaf-specific ferredoxins (*40, 43, 44, 117*). The control of gene transcription was light independent. This ferredoxin was found in all tissues as described earlier, but the others were found only in leaves. The amino acid sequences of these three ferredoxins were compared (*42*) and the homology between ferredoxins I and III was about 64% compared with 70–80% among leaf-specific ferredoxins from maize and other plants (*42*). Similar differences were previously reported for radish and spinach ferredoxins isolated from leaves and roots (*41, 114*). Thus, ferredoxins of the same tissues (leaf or root) in different plant species (maize and radish) are more similar to each other than those of leaf and root of the same plant species.

It is interesting to note that the N-terminal region of maize ferredoxin III (*115*) shows only one gap in the alignment, at position 12 (Fig. 2), as is found for radish root ferredoxin B-1 (*41*) (Fig. 2b). Other ferredoxins were in this respect similar to spinach leaf ferredoxin (Fig. 2a), indicating that the root and light-independent ferredoxins are structurally intermediate between those of light-dependent (or phototrophic) ferredoxins, which might have evolved later, and the cyanobacterial ferredoxins, probably the most primitive ones.

The unique structural characteristics described for radish white root ferredoxins (R-Fds) B-1 and B-2 suggested that such root- (or nonphotosynthetic tissue)-specific ferredoxins might exist in all other angiosperms (*41*). Although these iso-forms of ferredoxin could not clearly be distinguished in terms of electron-donating function to the ferredoxin-dependent enzymes so far examined, they may differ in electron-accepting function from NADPH via a ferredoxin-dependent oxidoreductase in nonphotosynthetic tissues (*41, 97*). An enzyme similar to ferredoxin-$NADP^+$ reductase was actually found in roots (*118*). This line of investigation was extended to spinach roots (*114*). Spinach root ferredoxin showed exactly the same structural characteristics at the N-terminal region as those of radish and maize root ferredoxins, as mentioned earlier; spinach root ferredoxin-$NADP^+$ reductase also showed a unique N-terminal sequence, having a shorter sequence at, and an insertion near, the N terminus, compared with the spinach leaf enzyme. What, then, is the functional difference between these iso-forms? As discussed above, functional difference is closely linked to tissue develop-

ment. Specific tissues have their specific functions; the possible roles of root ferredoxins have been proposed (*114*).

A ferredoxin-like electron carrier was also isolated from nongreen cultured tobacco callus cells, and donated electrons to nitrite reductase but it did not function in an $NADP^+$ photoreduction system (*119*). The absorption spectrum and molecular size were somewhat different from those usual for ferredoxins, and the N-terminal sequence was quite different (K. Wada, personal communication).

Several ferredoxin sequences are known for strains of *Synechococcus* (*74, 76–79*), a unicellular cyanobacterium. Unexpectedly, *Synechococcus* 6301 (*Anacystis nidulans*) ferredoxin resembled most closely ferredoxins of filamentous heterocystous cyanobacteria (*61, 62, 67, 72*). Moreover, another ferredoxin sequence identified in the same organism (*120*), based on its gene sequence, showed a marked difference, more than 54 residues, from the soluble ferredoxin in cell extracts, with an eight-amino acid extension at the C terminus compared with all others (*79*) (Fig. 2g). Furthermore, from the alignment given in Fig. 2, it shows consecutive gaps at positions 12 and 13 not found in other cyanobacterial ferredoxins. The C-terminal extensions, though only three or four residues, are present in *Halobacteria* ferredoxins as described below, but the sequences show little similarity. A hydropathy plot of the ferredoxin deduced from the gene sequence was significantly different from those of other ferredoxins, particularly at the region contributing to iron chelation, although major structural features in the three-dimensional structure of *S. platensis* ferredoxin (*87*) are probably preserved with some difference in surface charge distribution (*78*).

A functional difference of this ferredoxin was suggested (*79*), but detailed experimental evidence to support this are lacking since efforts to isolate the gene product were unsuccessful (*78*). Trace amounts of minor ferredoxins were reported for some cyanobacteria—*Spirulina maxima* (*100*), *Microcystis aeruginosa* (*121*), and *Aphanizomenon flos-aquae* (*121*)—and growth conditions affected the ratio of the iso-forms. The latter two cyanobacteria contained membrane-bound ferredoxins (*121*), and the hydropathy profile of the *Synechococcus* gene product suggests this might also be the case (*78*), but it cannot be excluded that this gene was a pseudogene. The N-terminal sequence of a membrane-bound ferredoxin isolated from *M. aeruginosa* was determined to be A-K-K-I-K-T-T-T- (*121*), which is unusual and suggests it might be an extension at the N terminus. Its absorption spectrum was also unusual (*121*), but in the absence of more detail the function and structure of this membrane-bound form remain enigmatic. Another membrane-bound ferredoxin, solubilized by detergent, was found in small quantity

in *A. flos-aquae,* but this ferredoxin showed similarity to those of the usual cyanobacterial ferredoxins. The physiological significance of this ferredoxin is unclear.

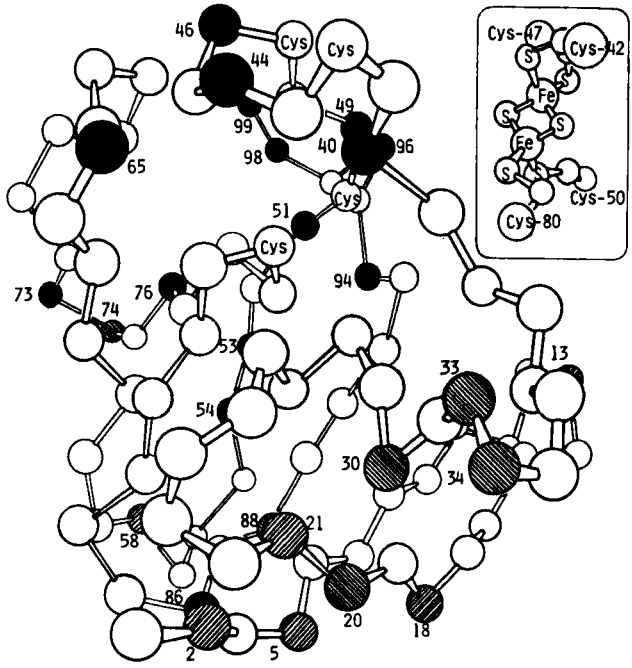
B. Reduction Potential Differences of Ferredoxin Iso-Forms

Two ferredoxins, I and II, isolated from a cyanobacterium, *Nostoc* strain MAC, showed a marked difference in midpoint reduction potential, −350 mV for ferredoxin I and −455 mV for ferredoxin II (*99, 100*), and also in their activities in $NADP^+$ photoreduction and the phosphoroclastic reaction system (*98, 122*). They have 34 amino acid differences, probably reflecting the different biophysical properties of the two ferredoxins (*69*). *Chlorogloeopsis fritschii* ferredoxin (*62*) and *Synechocystis* 6714 ferredoxin (*80*) showed very similar reduction potentials and enzymatic activities compared to those of *Nostoc* MAC ferredoxin I (*123*). Further, in amino acid sequence, *Chlorogloeopsis* and *Synechocystis* ferredoxins showed 18 and 25 amino acid differences from *Nostoc* ferredoxin I and 25 and 35 differences from *Nostoc* ferredoxin II, respectively, indicating the latter ferredoxin to be markedly different from the others. These distinctions were visualized on the three-dimensional structure model based on *S. platensis* ferredoxin (*69*) as shown in Fig. 3. Unique amino acid changes in each ferredoxin, when compared with the others, were identified and most of the unique changes found in *Nostoc* ferredoxin II were located near the Fe–S cluster within 10 Å of the cluster center. The low reduction potential of *Nostoc* ferredoxin II presumably results from the distribution of unique amino acid residues near the cluster, conferring its distinct biological properties (*69*).

C. Ferredoxins and Related Proteins in Organisms Other Than Oxygenic Photosynthesizers

1. Halobacterial Ferredoxins

Two ferredoxins with a [2Fe–2S] cluster were isolated from *Halobacterium halobium* (*124, 125*) and *Halobacterium* of the Dead Sea (*126*), extremely halophilic bacteria. Spectroscopic properties, the acidic nature of proteins, reduction potentials (−340 to −345 mV), and the contents of nonheme iron and inorganic sulfur atoms indicated that the proteins closely resembled those of chloroplast ferredoxins except that the molecular masses were larger, about 15 kDa (compared to 11 kDa (*124–126*). Immunological studies suggested the halophilic bacterial ferredoxins and chloroplast ferredoxins differed, but the bacterial ferre-

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    1          .          .          .          . ▽    ▽  ▽
(a) AT-YKVRLFNAAEGLDETIEVPDDEYILDAAEEAGLDLPFSCRSGSCSSC
(b) ATVYKVTLV-DQEGTETTIDVPDDEYILDIAEDQGLDLPYSCRAGACSTC
(c) AT-YKVTLINDAEGLNQTIEVDDDTYILDAAEEAGLDLPYSCRAGACSTC
(d) AS-YTVKLIT-PDG-ENSIECSDDTYILDAAEEAGLDLPYSCRAGACSTC
   51          .          .          ▽          .       99
(a) NGILKKGTVDQSDQNFLDDDQIAAGNVLTCVAYPTSNCEIETHREDAIA
(b) AGKIVSGTVDQSDQSFLDDDQIEKGYVLTCVAYPTSDLKIETHKEEDLY
(c) AGKIKSGTVDQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIETHKEEELY
(d) AGKITAGSVDQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIETHKEEDLY
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FIG. 3. Distribution of the unique residues in the three-dimensional structure of *S. platensis* ferredoxin (*86*). The inset shows the Fe–S cluster deleted from the main chain drawing. ●, ●, and ● represent the unique residues in *Nostoc* MAC ferredoxin II and I (*69*) and *Synechocystis* 6714 ferredoxin (*80*), respectively. *Spirulina platensis* ferredoxin has a gap at position 3 if aligned as shown in the comparison given below the structure, and the numbering on the three-dimensional structure is based on this alignment (*86*). The sequences are the ferredoxins from (a) *Nostoc* MAC II (*69*), (b) *Nostoc* MAC I (*86*), (c) *Chlorogloeopsis fritschii* (*62*), and (d) *Synechocystis* 6714 (*80*). A gap is inserted to maximize homology in the alignments. Italic letters indicate unique changes in residues in only one ferredoxin. An inverted triangle above the sequences indicates the cysteine residues chelating the two iron atoms.

doxins themselves showed 80% antigenic cross-reactivity (*127*). *Halobacteria* do not perform photosynthesis, nitrogen fixation, or hydroxylation reactions, and these ferredoxins would not mediate electron transfer reactions in $NADP^+$ photoreduction by chloroplasts (*125, 126*) or ferredoxin-dependent cytochrome *c* reduction mediated by ferredoxin-$NADP^+$ reductase (*126*). *Halobacteria halobium* ferredoxin would function as an electron carrier in α-ketoacid decarboxylation of α-ketoglutarate, pyruvate, and α-ketobutyrate, as would a cyanobacterial ferredoxin (see Ref. *128* and references therein). Another ferredoxin from *Halobacteria* of the Dead Sea functioned in nitrite reduction (*129*), as would algal and plant ferredoxins. An interesting finding with the ferredoxin from *Halobacteria* of the Dead Sea was that it accepted electrons from the photosystem on illumination, but would not participate *in vitro* in further transfer of electrons to ferredoxin-$NADP^+$ reductase (*130*). This was also observed for the *H. halobium* ferredoxin (T. Hase, personal communication, 1980). This observation implies that halobacterial ferredoxins have a conformation different from those of chloroplast ferredoxins and that they probably failed to interact with the ferredoxin-$NADP^+$ reductase in forming the ternary complex of ferredoxin, reductase, and $NADP^+$, as observed for chloroplast-type ferredoxins (*131–134*).

The amino acid sequences of these two halobacterial ferredoxins are known (*135, 136*) and that for *H. halobium* ferredoxin is given in Fig. 2h. Both ferredoxins have extensions of about 20 residues at their N termini and of 3 to 4 residues at their C termini, compared with those of chloroplast ferredoxins, making a total of 128 amino acid residues. The four cysteines essential for [2Fe–2S] cluster formation are arranged just as in chloroplast ferredoxins. There is a high degree of homology between halobacterial ferredoxins and chloroplast ferredoxins (*135, 136*). Halobacterial and chloroplast-type ferredoxin residues either identical to or similar to each other are mainly located near the functionally important cysteine residues (*135, 137*). The lysine residue at position 118 in both ferredoxins was acetylated; the biological significance of this is unclear. The N-terminal region contains many acidic residues (36–41% of the 22 residues) and no basic residues. This might be a consequence of the adaptation of the organism to life in high salt concentrations (*135, 137*), or these structural features might imply some specific function for the halobacterial ferredoxins.

The three-dimensional structure of ferredoxin of *Halobacterium* of the Dead Sea (*137, 138*) was elucidated to see whether the tertiary fold of the *Spirulina* ferredoxin (*87*) could be discerned in the electron density map of the halobacterial ferredoxin. Most of the electron density

profile, as expected from the sequence comparison (*102*), fitted well except an unusual feature interpreted as belonging to the N-terminal region of the halobacterial ferredoxin, which seemed to form a separate domain remote from the cluster (*137*). The [2Fe–2S] cluster region was quite similar to that of *Spirulina* ferredoxin and was not protected by any acidic residues (*137*). Refinement of these data is now in progress and preliminary analysis suggests that one of the β strands at the extreme N-terminal region found in *Spirulina* ferredoxin (*87*) is missing in the halobacterial ferredoxin, and that the acidic N-terminal region projects toward the Fe–S cluster region, although other regions are likely to be in a folding configuration similar to that of *Spirulina* ferredoxin (K. Fukuyama, personal communication, 1991). More detailed analysis will enable correlation of the unique structural features with the specific functions of halobacterial ferredoxins.

Comparison of the amino acid sequences of *Spirulina* ferredoxin (or any one of the other plant-type ferredoxins) and the halobacterial ferredoxins showed a marked difference at position 85, which is Tyr for *Spirulina* ferredoxin, Phe or Ile for the others, and Ser for the halobacterial ferredoxins. The side chain at this position is oriented toward the outside of the molecule; therefore, this region of the chloroplast ferredoxins would interact readily with residues on adjacent proteins (*87*), and probably with ferredoxin-$NADP^+$ reductase (*88, 95, 102, 103*), whereas halobacterial ferredoxin cannot interact with the reductase.

2. *Rhodobacter Ferredoxin*

A gene (*fdxC*) coding for a protein similar to the chloroplast-type [2Fe–2S] ferredoxins has recently been found in the nitrogen fixation gene (*nif* gene) clusters of *Rhodobacter capsulatus* SB1003, a photosynthetic purple nonsulfur bacterium (*139*). It was located just upstream of *fdxN* (Section IV,A), which encodes ferredoxin I, and the amino acid sequence deduced from its nucleotide sequence is given in Fig. 2i. A similar observation was also reported for a partial sequence of the corresponding *R. capsulatus* B10 gene (*140*), but with some differences. The sequence showed the four conserved cysteine residues, which would be able to chelate two iron atoms as in chloroplast-type ferredoxins. A comparison of this sequence and the sequence of horsetail or spinach ferredoxin showed that they had low but significant similarities to 24 or 18 corresponding residues, respectively (see Fig. 2). This sequence is also similar to those of [2Fe–2S] ferredoxin-like proteins that function in oxygenase systems (Fig. 2j), as described later. Aerobic nitrogen-fixing bacteria such as *Azotobacter vinelandii* must have mechanisms to protect the oxygen-sensitive nitrogenase when fixing nitrogen. *Azo-*

tobacter [2Fe–2S] ferredoxin (ferredoxin II), which is coinduced with the nitrogenase, has been assumed to function in protecting the oxygen inactivation of nitrogenase (*141*); we may speculate that any trace amount of oxygen is trapped by the oxygenase system to keep the environment of the nitrogenase oxygen free.

A mutant of *R. capsulatus* SB1003 with the *fdxC* gene engineered to eliminate the region near the Fe–S center with three cysteines would not grow on a medium containing no fixed nitrogen under illuminated anaerobic conditions; the wild-type bacteria grew rapidly under these conditions (*142*). The mutant grew on media with fixed nitrogen under either illuminated anaerobic or dark aerobic conditions. In conjunction with data from a complementation experiment, it was concluded that the *fdxC* gene product, a chloroplast-type [2Fe–2S] ferredoxin, is required for nitrogen fixation in *R. capsulatus* (*142*). A flavodoxin-like gene was found upstream of *fdxC* (*143*). A recent paper has reported that the *fdxC* gene was coexpressed with the ferredoxin I gene (*fdxN*) in *Escherichia coli* and that the product contained a [2Fe–2S] cluster (*144*), confirming a previously reported result (*142*). The protein was suggested to be involved in nitrogen fixation, which was in accord with the data obtained by the gene modification experiments mentioned above.

Other ferredoxin groups with a [2Fe–2S] cluster are found in several bacteria and also function in oxygenation and other reaction systems. These have been reviewed in detail elsewhere (*8, 9*), thus only a few groups are discussed in the following sections.

3. *Clostridium Ferredoxin*

A [2Fe–2S] ferredoxin was found in *Clostridium pasteurianum,* when it was grown with N_2, in addition to the typical 2[4Fe–4S] ferredoxin (see Refs. *8, 145,* and *146* and references therein). The magnetic properties and amino acid sequence (102 residues) of the [2Fe–2S] ferredoxin were different from those of other [2Fe–2S] ferredoxins. Figure 4 compares the sequences of several ferredoxins, emphasizing the distribution of the cysteine residues, some or all of which will chelate the two iron atoms, depending on the particular ferredoxin. The fact that the *C. pasteurianum* ferredoxin (Fig. 4a) was a dimer and contained two [2Fe–2S] clusters is rather unique (*146*). Although its function is not definitely assigned, it will transfer electrons in the hydrogenase system (*147*) and may show relatedness to nitrogen fixation genes (*146*). From the sequence the secondary structure could be predicted, providing a basis for suggesting that there were several ferredoxin groups that originated from different ancestors (*8, 9*). Another [2Fe–2S] ferredoxin

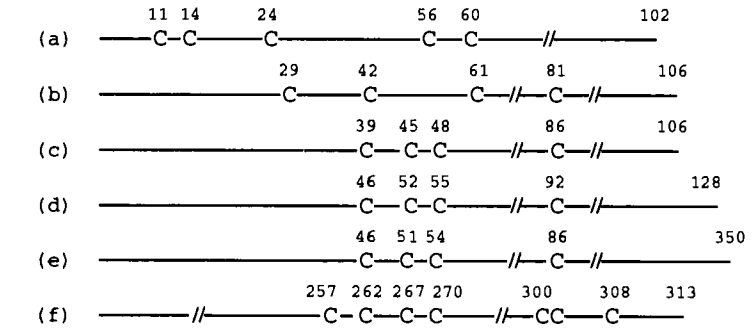


FIG. 4. Distribution of cysteine residues of various [2Fe–2S] ferredoxins and ferredoxin-like proteins. Cysteine residues (denoted by C) are the candidates for chelating the two iron atoms. (a) *Clostridium pasteurianum* [2Fe–2S] ferredoxin (*146*), (b) *Pseudomonas putida* ML2 protein B (*155*), (c) *P. putida* [2Fe–2S] ferredoxin (putidaredoxin) (*150*) (the four cysteine residues being assigned) (*151*), (d) bovine adrenodoxin (*157, 170–172*) (the four cysteine residues being assigned) (*158*), (e) *Pseudomonas* XylA protein (*161*), and (f) *Pseudomonas* VanB protein (*164*).

was isolated from a photosynthetic bacterium, *Rhodopseudomonas marina* (*148*), and proved similar to that of *Rhodopseudomonas palustris* (*149*) but was without marked resemblance to the clostridial [2Fe–2S] ferredoxin.

4. *Ferredoxins in Oxygenase Systems*

Pseudomonas putida, an aerobe, produces a [2Fe–2S] ferredoxin, putidaredoxin, which functions in the camphor methylene hydroxylase system (*150*). However, when the bacterium was grown on a medium containing various aromatic substrates, corresponding multicomponent oxygenating enzyme systems were induced with [2Fe–2S] ferredoxins of the putidaredoxin type (see Refs. *9* and *152* and references therein). Among them, a [2Fe–2S] ferredoxin, isolated as protein B from *P. putida* utilizing benzene as its sole carbon source, was found to function as an electron carrier in the benzene dioxygenase system (*153, 154*). The amino acid sequences of the ferredoxin from *P. putida* strain ML2 (106 residues) (*155*) and strain BE-81 (91 residues deduced from DNA sequencing) (*156*) showed that the former (Fig. 4b) has 15 additional residues at the N terminus compared to the latter, but there were only five amino acid differences throughout the rest of the polypeptide chain. Since the distribution of cysteine residues in the ML2 strain ferredoxin is completely different compared to putidaredoxin (*150*) (Fig. 4c) and adrenodoxin (*157*) (Fig. 4d), this ferredoxin comprises a new class. The

location of histidine near a cysteine was assumed to be responsible for the somewhat higher reduction potential of this ferredoxin (*155*).

A putidaredoxin-type [2Fe–2S] ferredoxin isolated from an anaerobic protozoan, *Trichomonas vaginalis,* was found to function in the pyruvate oxidation system (*159*); the sequence of this ferredoxin was closest to that of putidaredoxin (*160*).

Pseudomonas putida xylene monooxygenase consists of two nonidentical subunits and catalyzes the oxidation of toluene and xylene. The nucleotide sequences of the corresponding genes revealed that the deduced amino acid sequence of the XylA product (350 residues) showed a very high similarity, at the N-terminal 90 residues, to plant [2Fe–2S] ferredoxin sequences (*161*) (Figs. 2j and 4e). A further interesting fact is that the rest of the sequence was very similar to those of ferredoxin-$NADP^+$ reductases (*162, 163*). Therefore, this subunit, which supplies electrons to a second subunit that participates in hydroxylation of the substrate carbon side chain, was formed by the fusion of a plant ferredoxin gene and a ferredoxin-$NADP^+$ reductase gene (*161*). Similarly, the C-terminal region of the VanB component of vanillate demethylase (313 residues) (Fig. 4f) (*164*) and the N-terminal regions of the MmoC component (protein C) of methane monooxygenase (347 residues) (*165*) and a polypeptide-5 of phenol hydroxylase (353 residues) (*166*) have sequences similar to those of plant-type ferredoxins.

Mitochondrial adrenodoxin-type [2Fe–2S] ferredoxins functioning in cytochrome *P*-450-linked monooxygenase systems have been isolated from animal tissues: adrenocortex (adrenodoxin), ovary, thyroid, placenta, kidney (renoredoxin), liver (hepatoredoxin), brain, and others (see Ref. *167* and references therein, *168*).

The amino acid sequence of adrenodoxin was different from those of hepatoredoxin (*167*) and reno(re)doxin (*169*). In addition to adrenodoxin and reno(re)doxin, larger molecular species of about 1.5 kDa have been found in both porcine kidney and bovine adrenal mitochondria (*169*); these species were named Trp-ferredoxin because of the presence of tryptophan, not usually found in these proteins. Trp-ferredoxins isolated from both tissues were structurally similar to each other, but different from ferredoxins, adrenodoxin, and reno(re)doxin, of the same tissues. Further, Trp-ferredoxin was the major component in kidney, but the minor one in adrenal gland, indicating some functional distinction in different tissues, as discussed earlier for plant ferredoxins.

The amino acid sequence of bovine adrenodoxin (114 residues) as established initially (*157*) was later shown, when the encoding nucleotide sequence was established (*170–172*), to be of a partially degraded protein lacking 14 amino acids at the C-terminal region of the original

protein (Fig. 4d). A nondegraded adrenodoxin was later isolated (*173*), confirming that the mature adrenodoxin was composed of 128 residues. The amino acid sequence of porcine adrenodoxin is known from protein sequencing (*174*); the amino acid sequences of chicken (*175*) and human (*168, 176*) adrenodoxin have been determined from nucleotide sequencing. Two [3Fe–4S] ferredoxins functioning in an oxygenation reaction are also known and are mentioned in Section III,E.

5. *Proteins in Respiratory Chain Complexes*

Bovine heart mitochondrial complex II, succinate–ubiquinone oxidoreductase, is composed of four subunits; the larger two, 70 and 27 kDa, respectively, are the subunits of succinate dehydrogenase (SDH) (*177*). The 70-kDa subunit contains a covalently bound flavin. The enzyme has three Fe–S clusters, S-1, S-2, and S-3, distributed equally between the two subunits. Clusters S-1 and S-2 are of the [2Fe–2S] and [4Fe–4S] type and S-3 is of the [3Fe–4S] type, with these cluster structures having originally been assigned by mainly spectroscopic analyses (*178*). The enzymes were also isolated from several microorganisms: *Neurospora crassa* (*179*), *Bacillus subtilis* (*180*), *Micrococcus luteus* (*181*), *E. coli* (*182, 183*), and *Paracoccus denitrificans* (*184*). Amino acid sequence analyses of the subunits added further information about those amino acid residues, most probably cysteine residues, that held the Fe–S clusters, by comparing the cysteine distributions on the sequences (Fig. 5). Complete amino acid sequences deduced from DNA sequencing of the SDH genes of *E. coli* (*182, 183*), *B. subtilis* (*185*), and yeast (*186*), and partial sequences of SDH of several species, including mammals,

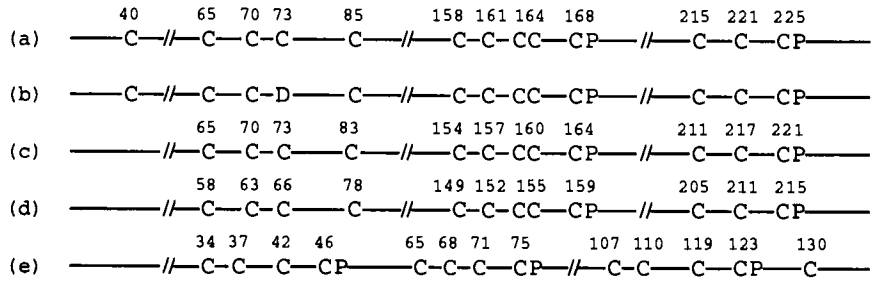


FIG. 5. Cysteine distributions of succinate dehydrogenase and fumarate reductase. Succinate dehydrogenase: (a) bovine (*188*) [yeast (*186*) shows essentially the same cysteine distribution as that of the bovine enzyme]; (b) *E. coli* (*183*) and (c) *Bacillus subtilis* (*185*). Those of human, rat, *Drosophila melanogaster, Arabidopsis thaliana,* and yeasts have been partially sequenced (*187*). Fumarate reductase: (d) *E. coli* (*190*) [*P. vulgaris* (*192*) shows essentially the same cysteine distribution as that of the *E. coli* enzyme] and (e) *P. vulgaris orfA*-encoded product (*192*).

insects, plants, and yeasts (*187*), as well as the results from direct protein sequencing of the bovine enzyme (*188*), are now available. The structure of fumarate reductase (FRD), the terminal reductase of an anaerobic respiration system, from *E. coli* (*189–191*), *Proteus vulgaris* (*192*), and *Wolinella succinogenes* (*193*) is similar in composition and cluster form to the structure of SDH (see Ref. *194* and references therein), and FRD and SDH sequences are also known to be similar.

Flavin-containing subunits of both SDH and FRD showed no apparent structural similarity to ferredoxins in terms of cysteine distribution; rather the cysteines were scattered along the whole sequences, leading to the suggestion that the three clusters in these enzymes may be held exclusively by the iron protein subunit (*178, 195*), since these showed very similar structural characteristics to those of ferredoxins in terms of cysteine distribution (Fig. 5). There are apparently three groups of cysteines, I to III. The arrangement of cysteines in group I is similar to those of the plant [2Fe–2S] ferredoxins (*105*), but *E. coli* SDH iron protein subunit has aspartic acid at the position corresponding to the third cysteine of cluster I. Therefore, the [2Fe–2S] cluster may be held by Cys 65, Cys 70, and two other cysteines, possibly Cys 85 and either another Cys from the iron protein subunit or one from the flavoprotein subunit. However, based on gene mutation analysis, the [2Fe–2S] cluster of *B. subtilis* SDH (*196*) is probably in the first cysteine group, I. For FRD, the [2Fe–2S] and [3Fe–4S] clusters are suggested to be entirely in the iron–protein subunit based on site-directed mutagenesis of the gene from *E. coli,* and the [4Fe–4S] cluster is probably in the flavoprotein subunit or bridges the two subunits (*197*). The [2Fe–2S] clusters of these two enzymes have reduction potentials and spectroscopic properties different from those of plant ferredoxins (*178, 197*). Another mutation experiment of *E. coli* FRD indicated that the four cysteine residues in group I are essential for the formation of a functional center 1, [2Fe–2S] cluster, but a noncysteinyl ligand cannot be excluded (*198*). Therefore, a final conclusion concerning the cluster awaits further results.

There are further proteins with [2Fe–2S] cluster(s) in respiratory and photosynthetic electron transfer systems, such as the Rieske Fe–S proteins of cytochrome bc_1 and b_6f complexes and the Fe–S proteins of complex I, NADH dehydrogenase (*177, 199*), but these are not reviewed here.

III. [4Fe–4S] and [3Fe–4S] Ferredoxins and Related Proteins

In this section we shall deal with a range of proteins with [4Fe–4S] or [3Fe–4S] clusters; a number of these, including aconitase (*7*), nitrite

reductase (*200*), sulfite reductase (*200*), trimethylamine dehydrogenase (*201*) and some others, are beyond the scope of this review, and some of the bacterial ferredoxins have been previously reviewed (*9, 10*).

A. Low-Potential Ferredoxins with a Single [4Fe–4S] or [3Fe–4S] Cluster

Several bacteria produce ferredoxins with only a single [4Fe–4S] or [3Fe–4S] cluster. These cluster types are interconvertible under certain conditions and the biological significance of the presence of a [3Fe–4S] cluster remains to be clarified (*9*), whether it occurs singly or in the [4Fe–4S][3Fe–4S] ferredoxins described later.

Bacteria with a single cluster include *Bacillus polymyxa* (*9*), several species of sulfate-reducing bacteria, *Desulfovibrio* (*9, 202*), the thermophile *Clostridium thermoaceticum* (*203*), *Clostridium formicoaceticum* (*204*), probably *Methanosarcina thermophila* (*205*), *Spirochaeta aurantia* (*206*), *Rhodopseudomonas marina* (*148*), the thermophiles *Bacillus stearothermophilus* (*207*) and *Bacillus thermoproteolyticus* (*208*), and a hyperthermophilic bacterium, *Pyrococcus furiosus* (*209*), which will be described in Section III,C. Their ferredoxins are variously involved in oxidoreduction reactions of bacterial nitrate reduction, sulfite reduction, CO_2 reduction, and pyruvate oxidation (*9*). Amino acid sequences of some of these ferredoxins are known: *Desulfovibrio gigas* [4Fe–4S] ferredoxin I, which is a trimer (*210*), and [3Fe–4S] ferredoxin II, which is a tetramer (*9* and references therein); *Desulfovibrio africanus* [4Fe–4S] ferredoxin I (*211*); *Desulfovibrio desulfuricans* Norway [4Fe–4S] ferredoxin I (*212*); *D. vulgaris* Miyazaki [4Fe–4S] ferredoxin II (*202*); *B. stearothermophilus* (*213*) and *B. thermoproteolyticus* [4Fe–4S] ferredoxins (*214*); and *C. thermoaceticum* [4Fe–4S] ferredoxin (*215*).

Figure 6 compares the sequences of ferredoxins with a single [4Fe–4S] cluster. Among them the three-dimensional structures of *D. gigas* ferredoxin (*216*) and *B. thermoproteolyticus* ferredoxin (*214, 217*) are known.

The two closely related thermophilic bacterial ferredoxins have 81 amino acid residues, but the others have about 60 residues. They have only four common cysteine residues chelating four iron atoms, which conform to one [4Fe–4S] cluster out of the two found in clostridial-type 2[4Fe–4S] ferredoxins, as shown in the three-dimensional structure of *Peptococcus aerogenes* ferredoxin (*218, 218a*). The apparent sequence homology observed in these [4Fe–4S] ferredoxins indicated that they must have a common tertiary structure, and also they might in part

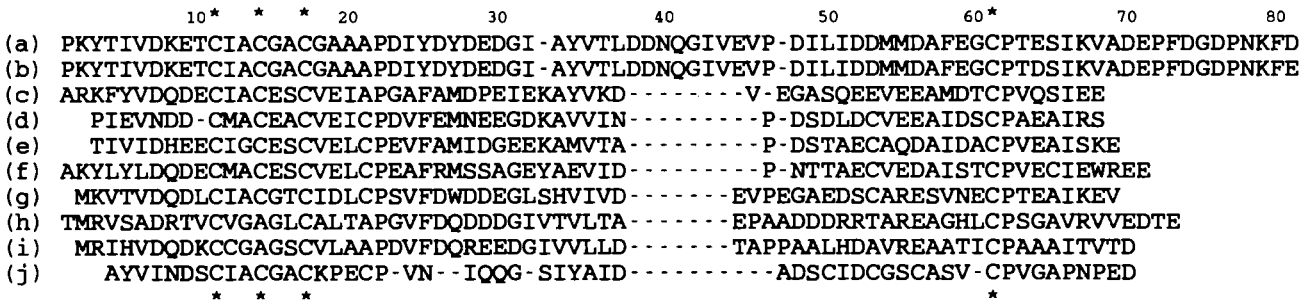
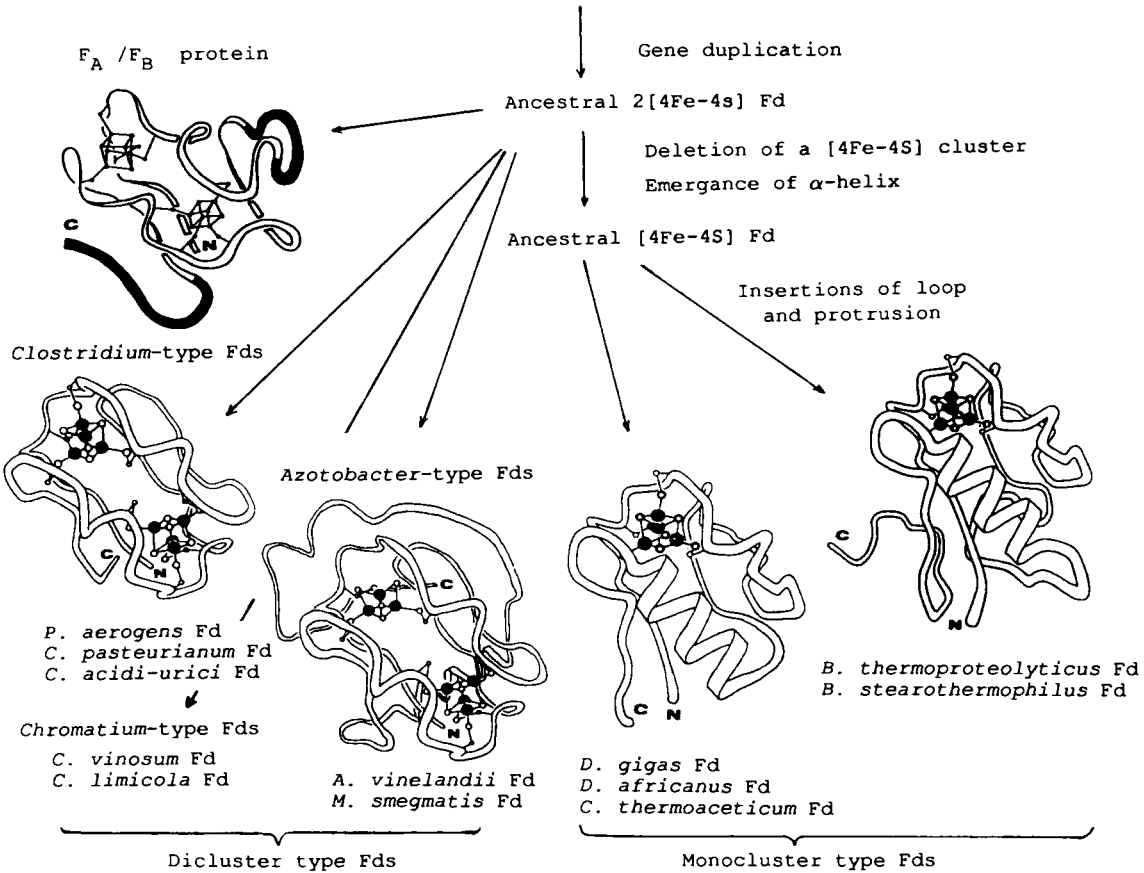


FIG. 6. Amino acid sequences of [4Fe–4S] and [3Fe–4S] ferredoxins. Position number is for the *Bacillus* ferredoxins. Gaps are inserted to align the sequences for maximum homology. Positions for cluster-binding cysteine residues are indicated by asterisks. (a) *Bacillus stearothermophilus* (*213*), (b) *Bacillus thermoproteolyticus* (*214*), (c) *Desulfovibrio africanus* I (*211*), (d) *Desulfovibrio gigas* I (*210*), (e) *Desulfovibrio desulfuricans* Norway I (*212*), (f) *Desulfovibrio vulgaris* Miyazaki II (*202*), (g) *Clostridium thermoaceticum* (*215*), (h) *Streptomyces griseolus* [3Fe–4S] ferredoxin 1 (*265*), (i) *S. griseolus* [3Fe–4S] ferredoxin 2 (*265*), and (j) *Peptococcus aerogenes* (i.e., *Peptostreptococcus asaccharolyticus*) 2[4Fe–4S] ferredoxin (*218a*).

have a folding similar to that of *P. aerogenes* ferredoxin, although the primary structures of these two classes are diverse except for the region around the first cluster of *P. aerogenes* ferredoxin. Figure 7 suggests evolutionary trends with model drawings of the tertiary structures of four of the bacterial ferredoxins (adapted from Refs. *214* and *219*) and one ferredoxin-like protein isolated from the photosynthetic reaction center (see Section IV,F). The [4Fe–4S] cluster of *B. thermoproteolyticus* ferredoxin is located at the top surface of the molecule and is geometrically identical to those of *P. aerogenes* ferredoxin (*218*). Hydrophobic residues surround the Fe–S cluster and the Ile 66, which is close to the cluster, is conserved in all [4Fe–4S] ferredoxins (Fig. 6).

The main chain topology of *B. thermoproteolyticus* ferredoxin with 81 residues is remarkably similar to that of *P. aerogenes* ferredoxin with 54 residues and the 2[4Fe–4S] clusters, and the conformations around the Fe–S clusters of *B. thermoproteolyticus* and the first cluster in *P. aerogenes* ferredoxin are superimposable. The NH—S hydrogen bonds and other segments of the upper half of the molecule are very similar to each other (*214*). The lower half of *B. thermoproteolyticus* ferredoxin molecule shows differences in topology and sequence from those of *P. aerogenes* ferredoxin, as seen in Fig. 7. The sequence Ile 51 to Gly 60 of *B. thermoproteolyticus* ferredoxin forms an α-helix, which corresponds to the second cluster of *P. aerogenes* ferredoxin, including Cys 53 and 56. A total of about 10 residues is inserted in *B. thermoproteolyticus* ferredoxin compared with the others and forms a loop structure, which can be deleted with three remaining residues without affecting helix formation (*214*). The C-terminal region of *B. thermoproteolyticus* ferredoxin has an extension of about 10 residues compared with the others. The other localized twofold symmetry observed in *P. aerogenes* ferredoxin (*218*) is not seen in *B. thermoproteolyt-*

FIG. 7. Evolutionary processes in bacterial ferredoxins and a chloroplast protein. Ferredoxins of the *Peptococcus* type (*218*) have two halves of a highly homologous sequence, each with a -C-X-X-C-X-X-C-X-X-X-C-P- sequence. *Chromatium* and *Chlorobium* ferredoxins have five to six residues instead of the two between the second and third Cys in the C-terminal half and have an extension at the C terminus (see also Fig. 9). Ferredoxins of the *Azotobacter* type (*310, 311*) have insertions similar to those of the *Chromatium* type and a large C-terminal extension. These have [4Fe–4S][3Fe–4S] clusters. Ferredoxins of the monocluster type have lost the second binding site in the sequence, which does not show any detectable internal duplication. The F_A/F_B protein isolated from the photosystem I complex (see Fig. 11 and Section IV,F) was simulated on the basis of the structure of *P. aerogenes* ferredoxin (*218*). This protein conserves the duplicated sequence with an insertion and C-terminal extension (*349*). Ferredoxins of the *Desulfovibrio* type (*216*) have a conformation similar to that of *B. thermoproteolyticus* ferredoxin (*214*).


Gene duplication
Ancestral 2[4Fe-4s] Fd
Deletion of a [4Fe-4S] cluster
Emergance of α-helix
Ancestral [4Fe-4S] Fd
Insertions of loop and protrusion
F_A /F_B protein
C
N
Clostridium-type Fds
Azotobacter-type Fds
P. aerogens Fd
C. pasteurianum Fd
C. acidi-urici Fd
Chromatium-type Fds
C. vinosum Fd
C. limicola Fd
A. vinelandii Fd
M. smegmatis Fd
D. gigas Fd
D. africanus Fd
C. thermoaceticum Fd
B. thermoproteolyticus Fd
B. stearothermophilus Fd
Dicluster type Fds
Monocluster type Fds

icus ferredoxin (*214*). That the aromatic residues are not essential for electron transfer but rather are, with the hydrophobic amino acid residues, crucial for stabilization of the Fe–S cluster has been discussed in detail (*214, 219*).

On the basis of sequence comparison and secondary structure prediction, the α-helix found in *B. thermoproteolyticus* ferredoxin was predicted to be present in other [4Fe–4S] ferredoxins (*214*). The four other ferredoxins were suggested to fold in a manner similar to that of *B. thermoproteolyticus* ferredoxin except for the loop and the C-terminal extension (*214*). This prediction proved correct for *D. gigas* ferredoxin II (*216*). Both ferredoxins I and II of *D. gigas* have different oligomeric forms. Ferredoxin I is in a trimeric state and II is in a tetrameric state, with a [4Fe–4S] and a [3Fe–4S] cluster per monomeric unit, respectively, although the amino acid sequences of both ferredoxins are identical (*9*). The physiological significance of an interconversion between ferredoxins I and II, with reduction potentials of -450 mV for I and -130 mV for II (*220*), has been discussed (*9*).

The three-dimensional structure of *D. gigas* ferredoxin II (*216*) revealed that its main chain fold was similar to that of *P. aerogenes* 2[4Fe–4S] ferredoxin (*218*), with a helical segment as predicted in the structural study of *B. thermoproteolyticus* ferredoxin (*214*) as given in Fig. 7. The presence of the α-helix in *D. sulfuricans* Norway ferredoxin was also predicted by NMR spectroscopy (*221*). The [3Fe–4S] cluster was similar to the one found in aconitase (*7*) and the three irons were chelated by Cys 8, 14, and 50, but not by Cys 11, forming a cluster just like a [4Fe–4S] structure but lacking one iron atom. Cys 11 was rotated and the side chain extended toward the solvent and was interestingly modified by $S(CH_3)_{1-2}$, this perhaps having some physiological significance (*216*). A simple rotation of the Cys 11 side chain so as to chelate the fourth iron necessary to form a [4Fe–4S] cluster would require a readjustment of the polypeptide chain around this region (*216*).

Bacillus stearothermophilus and *B. thermoproteolyticus* ferredoxins and *D. africanus* ferredoxin I have only the four cysteine residues necessary to construct a [4Fe–4S] cluster, but ferredoxins from *D. gigas, D. desulfuricans* Norway, and *C. thermoaceticum* have six cysteine residues. In place of the extra two cysteine residues the former three ferredoxins have alanine and an acidic residue, respectively. Side chains at these positions are close together, leading to the likelihood of disulfide bond formation between Cys 18 and Cys 42 in the case of *D. gigas* ferredoxin (*217, 222*), as confirmed by the X-ray analysis (*216*). An earlier chemical study of *D. gigas* ferredoxin had suggested a disul-

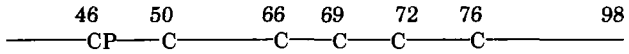
fide bond might be present in this molecule (*223*). The significance of the disulfide bridge is uncertain.

In their tertiary structure, *C. pasteurianum* ferredoxin and closely related 2[4Fe–4S] ferredoxins conserve the duplicated form of an ancestral half molecule in a twofold symmetry (Fig. 7). Other structures are variations of this basic form. The deletion of the second [4Fe–4S] cluster that is present in 2[4Fe–4S] ferredoxins was followed by the proximal peptide segment changing in conformation to an α-helix, maintaining a stable tertiary structure. Once it was introduced, the helix remained during evolution and only the N-terminal half segment has been conserved as the sequence holding an Fe–S cluster, corresponding to that present in the ferredoxins of more primitive fermentative bacteria such as *Clostridium*. As revealed by the comparison of *B. thermoproteolyticus* ferredoxin and *D. gigas* ferredoxin, the loop and protruding region found in the former were probably introduced after divergence of *B. thermoproteolyticus* ferredoxin and other [4Fe–4S] ferredoxins (*214, 219*).

Papers dealing in more detail with the evolutionary aspects of bacterial ferredoxins have appeared previously (*224–227*).

B. Ferredoxin-Like Proteins in Symbiotic Nitrogen-Fixing Bacteria

Genes coding for ferredoxin-like proteins, designated as *fixX*, have been found through molecular genetic studies of the root nodule symbiotic nitrogen-fixing bacteria, *Rhizobium meliloti* (*228–230*), *Rhizobium trifolii* (*231*), *Rhizobium leguminosarum* biovar *viciae* (*232*), *Bradyrhizobium japonicum* (*233*), and *Azorhizobium caulinodans* (*234*). Their products are composed of 98 residues, except the protein from *A. caulinodans* (97 residues), which lacks the N-terminal residue. As an example the sequence of *R. trifolii fixX*-encoded protein showing the cysteine distribution is



This protein has a single [4Fe–4S] cluster probably chelated by Cys 46, 66, 69, and 72, corresponding to the cysteine clusters of clostridial 2[4Fe–4S] ferredoxin but lacking the three cysteines near the N terminus, which, with one cysteine near the C terminus corresponding to Cys 76 in this sequence, forms one Fe–S cluster. They have the considerably longer N-terminal sequences than those of the common bacterial ferre-

doxins. To date, genes of this type have been only found in symbiotic nitrogen-fixing bacteria. The *R. meliloti fixX*-encoded product was reported to be essential for nitrogen fixation, a conclusion based upon transposon mutagenesis (*229*) and complementation (*228*) studies. All *fixX* genes except that in *B. japonicum* are located downstream of *fixABC,* which are required for symbiotic nitrogen fixation, and are cotranscribed from a promoter upstream of *fixA*. In *B. japonicum, fixX* is downstream of *fixBC,* and *fixA* is separate.

C. A Highly Thermostable [4Fe–4S] Ferredoxin

Several thermostable ferredoxins have been isolated from thermophilic bacteria (*9, 235, 236*) and cyanobacteria (*61, 237*). The major participation of external salt bridges linking residues near the N terminus to others near the C terminus was suggested to be responsible for the thermal stability of these bacterial ferredoxins (*238*); these ferredoxins also had increased hydrophobic interactions and hydrogen bond networks (*235, 239*) compared to other ferredoxins, which were less thermostable. Crucial salt bridges were also suggested to be responsible for the thermal stability of a cyanobacterial ferredoxin (*76*). A remarkably thermostable ferredoxin has been isolated from the hyperthermophilic, anaerobic archaebacterium, *Pyrococcus furiosus,* which grows optimally at over 100°C in submarine volcanic areas (*209, 240*). The ferredoxin, which donates electrons to hydrogenase, was purified under anaerobic conditions and was stable in 20% sodium dodecyl sulfate (SDS) or after incubation at 95°C for 12 hr. The reduction potential was −345 mV at pH 8.0. The molecular weight of this ferredoxin was about 7500, with four iron and four inorganic sulfur atoms and five cysteines, indicative of the presence of a [4Fe–4S] cluster. However, one of the four iron atoms was chelated by an aspartic acid and not a cysteine. The cysteine cluster was shown to be

```
   11  14  17  21          48      56    66
—C—C—C—CP—/ /—C——CP——
```

The interconversion between a [4Fe–4S] and [3Fe–4S] cluster was observed, but only the [4Fe–4S] with an aspartic acid coordinate cluster was thought to be present *in vivo*. A very recent report has shown that one of the four iron atoms in the native state of this ferredoxin is reactive with cyanide as an exogenous ligand (*241*).

D. High-Potential Iron–Sulfur Proteins

A group of proteins with a single [4Fe–4S] cluster showing a high reduction potential at around +340 mV was found in various photosyn-

thetic bacteria, including *Chromatium vinosum* (*242*), *Rhodospirillum salinarum* (*243*), *Thiocapsa roseopersicina* (*244*), *Rhodopseudomonas marina* (*148*), and others, except for *Ectothiorhodospira halophila* (*245*) and the denitrification bacterium, *Paracoccus* sp. (*246, 247*); these proteins are called high-potential iron–sulfur proteins (HiPIPs). The molecular weights are less than 10,000 and they are thought to function as electron carriers in photosynthesis (*248, 249*), anaerobic metabolism (*250*), and thiosulfate oxidation (*251*), although definite functions remain to be clarified. Amino acid sequences of 10 HiPIPs are known (*245, 247, 252–257*), and they are quite different from those of other ferredoxins (Fig. 8). The sequences currently available are summarized elsewhere (*245* and *257*), but the *Chromatium* HiPIP sequence (*254*) is given below to illustrate the distribution of the cysteine residues and of other amino acid residues conserved in 10 HiPIPs.

```
1        19          43    46        63        75   77 85
————————Y————————C————C————————C————————G—C—
```

Except for the four cysteine residues, which are necessary for chelating the four iron atoms, there are not so many conserved residues among HiPIPs as for low-potential ferredoxins. The four cysteines are distributed differently compared with those of low-potential ferredoxins. The evolutionary implications of the sequence anomaly of the HiPIPs have been described (*257*). Among the 10 HiPIPs, two iso-forms, I (120 mV) and II (50 mV), from an extremely halophilic purple phototrophic bacteria, *Ectothiorhodospira halophila,* were isolated and sequenced (*245*). There is 72% identity between the two iso-forms, and the four cysteines (Cys 43, 46, 63, and 77 using the *C. vinosum* HiPIP numbering) and two other residues (Tyr 19 and Gly 75) are conserved. The importance of these residues with particular regard to the reduction potential and hydrogen bonding in the three-dimensional structure is of interest. The sequence comparison suggested that *E. halophila* HiPIP iso-forms were most remote from the others (about 30% identity), indicating the divergence of these two at equal rates after gene duplication without evident change in function (*245*). This phenomenon may be different from those observed in various photosynthetic [2Fe–2S] ferredoxins (Section II,A). As they become available the sequences of HiPIP iso-forms found in other closely related bacteria should clarify the relationship between gene duplication and speciation (*245*). The three-dimensional structure of *C. vinosum* HiPIP is known (*258–260*) and the cluster geometry was shown to be essentially the same as those of the 2[4Fe–4S] from *P. aerogenes* ferredoxin (*261, 262*). Two

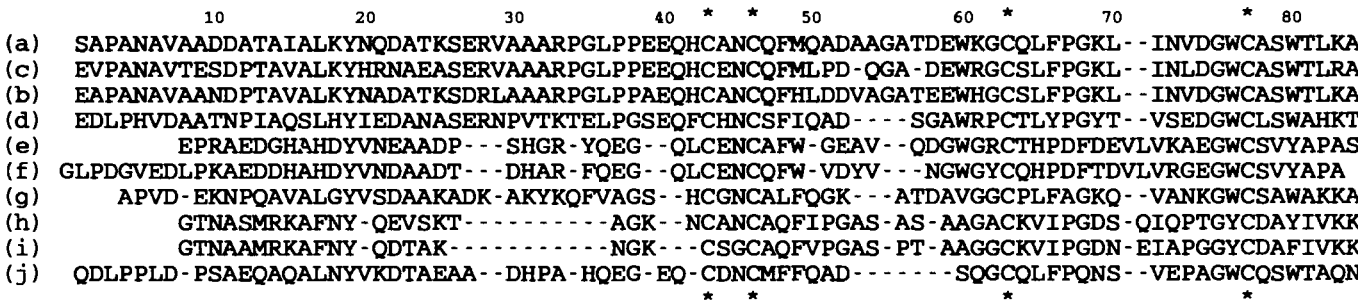


FIG. 8. Amino acid sequences of HiPIPs. Position number is for *Chromatium vinosum* protein. Gaps are inserted to align the sequences for maximum homology. Positions for cluster-binding cysteine residues are indicated by asterisks. (a) *Chromatium vinosum* (*10, 11*), (b) *Chromatium gracile* (*252*), (c) *Thiocapsa roseopersicina* (*252*), (d) *Thiocapsa pfennigii* (*255*), (e) *Ectothiorhodospira halophila* I (*245*), (f) *E. halophila* II (*245*), (g) *Rhodopseudomonas gelatinosa* (i.e., *Rhodocyclus gelatinosus*) (*256*), (h) *Rhodospirillum tenue* 3761 (i.e., *Rhodocyclus tenuis*) (*253*), (i) *R. tenue* 2761 (*257*), and (j) *Paracoccus* sp. (*247*).

preliminary crystallographic analyses have been carried out for *Rhodospirillum tenue* (*263*) and *E. halophila* (*264*) HiPIPs.

E. Ferredoxins with a Single [3Fe–4S] Cluster in Oxygenase Systems

In general, ferredoxins functioning in oxygenase systems are of the [2Fe–2S] form with sequences similar either to those of plant-type ferredoxins or to putidaredoxin, as described in Section II,C,4. In contrast, two new types of ferredoxins having a [3Fe–4S] cluster were found to be components of the *Streptomyces griseolus* monooxygenase system induced by a sulfonylurea herbicide (*265*). The corresponding genes encoding ferredoxin 1 and 2 were sequenced and the deduced amino acid sequences, 68 and 64 residues, respectively, showed 52% identity to each other (Fig. 6h and 6i, respectively). The spectral properties and iron and sulfur contents of both ferredoxins suggested that they each contained a single [3Fe–4S] cluster. Ferredoxin 1 has only three cysteines with the motif -C-X-X-A-X-X-C---CP-, and ferredoxin 2 has four cysteines with the motif -C-C-X-A-X-X-C---CP-, compared with [4Fe–4S] ferredoxins (Section III,A).

F. Proteins with a Single [4Fe–4S] Cluster Bridging Two Subunits

1. Photosystem I Reaction Center X Proteins

Oxygenic photosynthetic organisms have two photosystems (PSs), I and II. The oxidation of water with evolution of oxygen is achieved by PS II to produce a weak reductant whereas PS I produces a strong reductant able to reduce $NADP^+$. The two systems are linked by an electron transfer complex, the cytochrome b_6f complex that translocates protons (*266, 267*). The PS I complex is a multisubunit complex in the thylakoid membranes, which has six intrinsic electron transfer components enabling a series of oxidation–reduction transfers from plastocyanin to the [2Fe–2S] ferredoxin. Among them there are three Fe–S centers, X, A, and B. The Fe–S center X has a very low midpoint reduction potential, -705 mV, and transfers electrons from the chlorophyll primary electron donor, P-700, through A_0 and A_1 to the Fe–S center F_A/F_B protein (see Section IV,F). Various lines of evidence suggest that center X is a [4Fe–4S] cluster (*268*) bridged by two nonidentical but homologous proteins with the leucine zipper motif (*269*); these proteins are called core proteins I and II and have molecular masses of

about 64 kDa. The amino acid sequences of the two proteins of several organisms, ranging from higher plants to cyanobacteria, were deduced from the sequences of the corresponding genes (*psaA* and *psaB*) (*266*). The distribution of the four cysteine residues necessary for chelating the four iron atoms are completely different from those in other Fe–S proteins and were located in homologous positions in each subunit (*266*).

2. *Fe Proteins in Nitrogenase and Related Proteins of Chloroplasts and Cyanobacteria*

Nitrogenase is found only in prokaryotes and catalyzes the reduction of N_2 to NH_3. It consists of two components, the iron(Fe) protein and molybdenum–iron (Mo–Fe) protein (*270*). The enzyme requires ATP and a low-potential reductant, such as ferredoxin or flavodoxin, to reduce N_2. The Fe proteins have molecular masses of about 58–73 kDa, depending on source, and exist as homodimers. They function as a reductase with a reduction potential upon binding ATP of about -400 mV during electron transfer to the Mo–Fe protein. This has a [4Fe–4S] cluster per dimer, forming a V shape with the Fe–S cluster at the junction highly exposed to solvent (*271*). The cysteine residues chelating four iron atoms are thought to be located at positions 97 and 132 in both subunits of the protein from *A. vinelandii*. The distribution is completely different from those of other Fe–S proteins, including ferredoxins (*270*).

When the complete nucleotide sequences of chloroplast DNAs from liverwort, *Marchantia polymorpha* (*272*), and tobacco, *Nicotiana tabacum* (*273*), were established in 1986, a notable difference was found between the two in the gene organization and the amino acid sequences of the putative Fe–S proteins. Liverwort chloroplast DNA has three genes, *frxA*, *frxB*, and *frxC*, but tobacco has only two: *psaC*, corresponding to *frxA*, and *orf167*, corresponding to *frxB*. The *frxA* and *frxB* genes have cysteine distributions similar to those of 2[4Fe–4S] ferredoxins, but the *frxC* gene has a cysteine distribution similar to those of the nitrogenase Fe protein encoded by the *nifH* gene (*274*), which codes for a polypeptide composed of 289 amino acid residues. Nitrogen fixation is a feature found only in prokaryotes such as bacteria and cyanobacteria, and it was therefore suspected that the *frxC* gene product might have a function in a system other than nitrogen fixation (*275*). Incidentally, the *frxC* gene does not form an operon with the other structural genes (*nifD* and *nifK*) for nitrogenase, as is the case for the *nifH* gene (*276*), and no nitrogenase activity was detected in either the whole cells or intact chloroplasts of liverwort (*275*). The *frxC* gene product was

immunochemically detected in the soluble fraction of liverwort chloroplasts and found to be present as a dimer (molecular weight of about 67,000) (*275*) analogous to the Fe protein of nitrogenase (*274*). Further, the protein had a consensus ATP-binding sequence, Gly-X-X-X-X-Gly-Lys-Thr(Ser)- (*277*) at the N-terminal region and would bind to an affinity column of Cibacron Blue. The distribution of cysteine residues necessary to form a [4Fe–4S] cluster (*274, 278*) were conserved in the sequence. All these lines of evidence indicate that the *frxC* gene product may function, like the Fe protein in nitrogen fixation.

The *nifH* genes are highly conserved among nitrogen-fixing organisms generally, with about 60% homology or more in amino acid sequence (*279*), though the *frxC* gene products of liverwort and *Plectonema boryanum,* a filamentous cyanobacterium fixing nitrogen, showed only about 35% homology with any of the Fe proteins (*274, 280*). Interestingly, the *frxC* protein showed about 44% homology with a putative protein encoded by the *F202* gene (*281, 282*) located in the photosynthetic gene cluster (*282*) of *Rhodobacter capsulatus*. Also, the *F202* gene showed a low homology with the *nifH* genes (*281*). Later, the authentic *nifH* gene of the same bacteria was sequenced and was found to be highly homologous with other *nifH* genes (*283*). Taken in conjunction, this circumstantial evidence suggests that the proteins encoded by the two genes (*frxC* and *F202*) function in a common novel manner other than in nitrogen fixation.

Plectonema boryanum has genes corresponding to both *frxC* and *nifH,* and their putative proteins showed about 83% homology with the *frxC*-encoded protein of liverwort and about 84% with the *nifH*-encoded Fe protein of *Anabaena* PCC7120, yet these two genes in *P. boryanum* showed only 34% homology (*280*). The *P. boryanum frxC*-encoded putative protein product also has the ATP-binding sequence motif and the cysteine residues necessary to form a [4Fe–4S] cluster. After duplication of an ancestral gene, it seems that the *frxC* and *nifH* genes diverged into two separate groups and evolved independently to function in different metabolic systems. Figure 9 compares the distributions of cysteine residues implicated in forming the [4Fe–4S] cluster and the ATP-binding sequences deduced from consideration of the *frxC* and *nifH* gene sequences.

The function of the *frxC* gene product was unknown and it was anticipated that it might be concerned with a specialized metabolic role in lower plants and algae. A mutant of *P. boryanum* lacking the *frxC* gene has recently been obtained by targeted gene disruption mutagenesis, and another mutant lacking the *nifHD* genes necessary for nitrogen fixation has also been prepared (*289*). As expected, the latter mutant

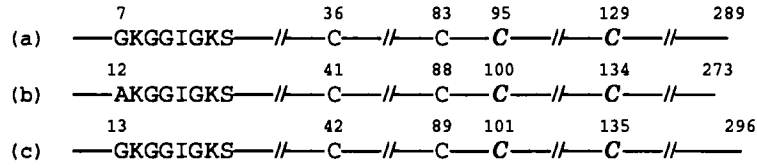


FIG. 9. Distributions of cysteine residues and ATP-binding motif in the sequences of *frxC*- and *nifH*-encoded proteins. Cysteine residues suggested to chelate iron atoms are italicized. Cysteine residues commonly found in all sequences are also given. (a) *Marchantia polymorpha* (liverwort) *frxC*-encoded protein (289 residues) (*284*) and *P. boryanum frxC*-encoded protein (286 residues) (*280*); (b) *R. capsulatus F202*-encoded protein (*281*); (c) *P. boryanum nifH*-encoded protein (296 residues) (*280*) and *Anabaena* 7120 *nifH*-encoded protein (299 residues) (*285*).

could not grow on a nitrogen-free agar plate but the former grew normally, regardless of the presence or absence of nitrogen sources, indicating the *frxC*-encoded protein is probably not essential for photosynthesis or nitrogen fixation. The *frxC* mutant grew almost at the same rate as the wild-type alga under dark conditions, but the chlorophyll content was greatly decreased. This finding is in agreement with the observations that cyanobacteria contain significant amounts of chlorophyll even when they are grown in the dark, in contrast to higher plants and algae, which have only the light-dependent chlorophyll biosynthetic system (*286, 287*). Liverwort similarly possessed chlorophyll even in a dark-grown culture. The light-independent reduction of protochlorophyllide *a* to chlorophyllide *a* has been demonstrated in a *Synechococcus* strain (*288*). These observations suggest that the *frxC*-encoded protein functions in an electron transfer reaction between protochlorophyllide *a* and chlorophyllide *a* (*280, 289*).

G. Succinate–Fumarate Oxidoreductases

As mentioned in Section II,C,5, succinate–fumarate oxidoreductase contains both [4Fe–4S] and [3Fe–4S] clusters that are probably formed by the cysteine clusters of the sequence motifs: -C-X-X-C-X-X-C-X-X-X-C-P- for cluster S-2 and -C-X-X-X-X-X-C-X-X-X-C-P- for cluster S-3, respectively (*178*) (Fig. 5).

IV. 2[4Fe–4S] and [4Fe–4S][3Fe–4S] Ferredoxins and Related Proteins

Since the observations in 1962 regarding bacterial and plant ferredoxins, many 2[4Fe–4S] and several [4Fe–4S][3Fe–4S] ferredoxins and

related proteins have been isolated and characterized. Bacterial ferredoxins with these clusters have been critically reviewed (*9, 10*) and here we deal only with a few special cases—in particular, several ferredoxins with specialized functions in one organism are discussed in relation to their structures. (Hydrogenases are reviewed by Voordouw, this volume.)

A. Photosynthetic Bacterial and *nif*-Related Fereredoxins

Ferredoxins isolated from the photosynthetic bacteria *Chlorobium thiosulfatophilum, Chlorobium limicola* I and II, *Chromatium vinosum,* and *Rhodopseudomonas palustris* I were classified in a phylogenetic subgroup of the 2[4Fe–4S] ferredoxins (*9*). They have sequences with two Cys clusters. The first group has the sequence -C-X-X-C-X-X-C-X-X-X-C-P-, typical of clostridial ferredoxins, whereas the second Cys clusters have a longer spacing of seven to eight residues between the second and third Cys rather than the typical spacing of two residues. They have also an extra (fifth) Cys (Fig. 10). There are two exceptions to this "photosynthetic bacterial" classification. One recently characterized exception is *R. capsulatus* ferredoxin II, which is of the [4Fe–4S][3Fe–4S] type and very similar to ferredoxin I from *A. vinelandii* (*142, 295–297*). The occurrence of ferredoxins of this type should be common to other members of Rhodospirillaceae, as is suggested for *R. rubrum* ferredoxin II from its physicochemical properties (*298*) and N-terminal sequence (K. Saeki and N. Nagai, unpublished results, 1990). Another puzzling exception is an extracellular ferredoxin purified from the culture medium of *R. rubrum,* which has a sequence closely similar to those of the clostridial ferredoxins (*299*). Since no similar ferredoxin has been reported from any other photosynthetic bacteria, genetic evidence is necessary to ascertain whether this ferredoxin is really encoded by the *R. rubum* genome.

Among the members of this subgroup, only the *R. capsulatus* ferredoxin I gene (*fdxN*) has been cloned and studied in terms of its molecular biology (*140, 144, 295*), and this work is summarized below.

As a consequence of molecular biological studies on genes related to nitrogen fixation, six ferredoxin-like genes have been identified. These encode products that possess structural features similar to those of the photosynthetic bacterial ferredoxins mentioned above (Fig. 10). Three of them, found in *R. meliloti* (*fdxN*) (*300, 301*), *B. japonicum* (*frxA*) (*302*), and *A. vinelandii* (*303*), were located just downstream of and probably cotranscribed with *nifB,* whose product is concerned in Mo–Fe protein maturation. The *B. japonicum frxA*-encoded product has Leu

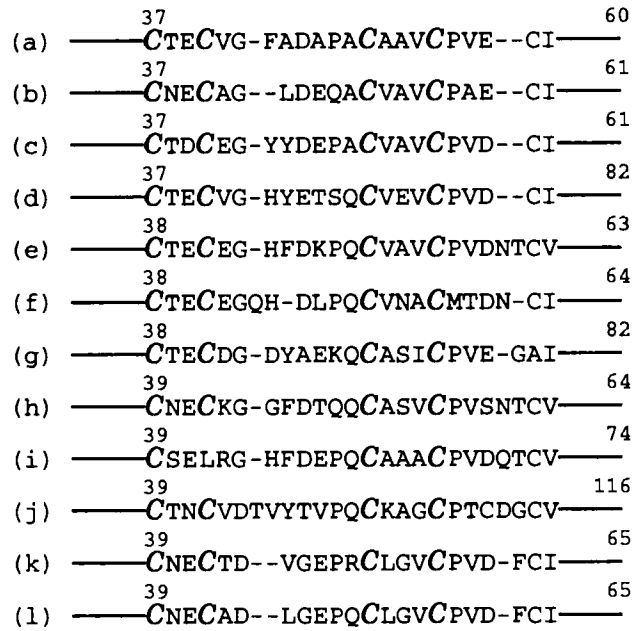


FIG. 10. Amino acid sequences of the second Cys clusters of photosynthetic and *nif* gene related ferredoxins. A gap is inserted to align the sequences for highest homology. Cysteine residues suggested to chelate the iron atoms are italicized. (a) *Chlorobium thiosulfatophilum* (*290*), (b) *Chlorobium limicola* I (*291*), (c) *C. limicola* II (*292*), (d) *Chromatium vinosum* (*293*), (e) *Rhodopseudomonas palustris* I (*294*), (f) *Rhodobacter capsulatus* (*fdxN*-encoded Fd I) (*140, 144, 295*), (g) *A. vinelandii* (*nifB* related) (*303*), (h) *Rhizobium meliloti* (*fdxN*) (*300, 301*), (i) *Bradyrhizobium japonicum* (*frxA*) (*302*), (j) *Anabaena* PCC7120 (*300*), (k) *A. vinelandii* (*vnfH* related) (*305*), and (l) *A. chroococcum* *nifH** related (*304*).

at the position, whereas its counterparts have the sixth Cys residue (*302*) (Fig. 10i) and an *A. vinelandii orf*-encoded product lacks the ninth Cys residue (*303*) (Fig. 10g). Another gene, *Anabaena* PCC7120 *fdxN*, is located upsteam of *nifB* (*300*) (Fig. 10j). It is interesting that this gene in vegetative cells is split into two parts separated by 55 kbp of DNA, and becomes an entity only after excision of the DNA during genome rearrangement accompanying heterocyst formation (*300*). In addition, *R. capsulatus fdxN* is also located in one of the major *nif* gene clusters in the bacterium but not adjacent to *nifB* (*140, 142*). The other two genes were found as ORFs downstream of the *nifH* homologue (*vnfH*) of the alternative nitrogenase system in *A. chroococcum* (*304*) and *A. vinelandii* (*305*) (Fig. 10, l and k, respectively).

Gene-specific disruption experiments have been carried out on three of the ferredoxins mentioned above. Interposon mutation of *B. japonicum frxA* was reported to have little affect on symbiotic nitrogen fixation, but decreased the nitrogenase activity of free-living cells to approximately 50% (*302*). *Rhizobium meliloti fdxN* was demonstrated to be necessary for symbiotic nitrogen fixation by complementation experiments on mutants; *fdxN*-depleted mutants were unable to fix N_2, but the ability was restored by plasmids with *fdxN* (*301*). Such disruption following complementation also showed that *fdxN* of *R. capsulatus*, a nonsymbiotic bacterium, was an important component for nitrogen fixation (*142*).

Because most of the known photosynthetic bacteria fix nitrogen (*306*), it might be speculated that all the 12 ferredoxins of the group in Fig. 10 have some specific role in the nitrogen fixation system and the unique longer insertion between the sixth and seventh Cys residues might have some functional significance in interacting with other component(s) of the system.

B. *Azotobacter*-TYPE [4Fe–4S][3Fe–4S] FERREDOXINS

The [4Fe–4S][3Fe–4S] ferredoxins represented by *A. vinelandii* ferredoxin I have two unique structural features and have been classified into a distinct phylogenetic group (*9*). One of the features is that the first Cys cluster is unique, with the sequence -C-X-X-X-K-X-X-X-C-X-X-X-C-P-V-X-X-, where X is often but not necessarily always C; the second Cys group is the usual sequence -C-X-X-C-X-X-C-X-X-X-C-P-. The other notable feature is the long C-terminal extension of 30 to 50 residues compared to that of the typical clostridial ferredoxins. Since a previous review (*9*), three additional members, the ferredoxins from *Pseudomonas stutzeri* (*307*), *Streptomyces griseus* (*308, 309*), and *R. capsulatus* (*142, 295–297*), have been added to the group.

The three-dimensional structure of *A. vinelandii* ferredoxin I has been redetermined (*310, 311*) (see Fig. 7) and refinement to 1.9 Å resolution (*312*) has enabled revision of the previous model (*313*). The geometry of the 3Fe cluster is essentially the same as that of the [3Fe–4S] cluster for *D. gigas* ferredoxin (*216*) and aconitase (*7, 314*), i.e., as in a [4Fe–4S] cluster but with one Fe atom removed, and completely different from the previous [3Fe–3S] model. The ligands for the [3Fe–4S] cluster were Cys 8, 16, and 49, whereas those of the other accompanying [4Fe–4S] cluster were Cys 20, 39, 42, and 45. Cys residues corresponding to these positions are conserved in all the ferredoxins of this type. In this group, the residue corresponding to the nonli-

gand Cys 11 is Val in *Mycobacterium* (*Micrococcus*) *smegmatis* (*315*), *Thermus thermophilus* (*316*), and *S. griseus* (*309*) ferredoxins and Glu in *Bacillus acidocaldarius* (*317*) ferredoxin, and that corresponding to Cys 24 is Ala in *B. acidocaldarius* (*317*). Superposition of the main chain topologies of *A. vinelandii* ferredoxin with 106 residues and of *P. aerogenes* 2[4Fe–4S] ferredoxin with 54 residues showed a striking similarity within residues 1–58 of the former, except for the insertion of additional residues 9, 10, 29, and 30 (*312*). The extended C-terminal portion of the former ferredoxin, residues 59 to 106, is in contact with the expanded loops, which contain these four extra residues and contribute a significant feature in the tertiary structure.

Although it was known that the two clusters of ferredoxins of this type have considerably different reduction potentials, there were discrepancies among the actual values reported. Recently, a direct electrochemical characterization using pyrolytic graphite edge electrodes has revealed that *A. chroococcum* ferredoxin I, a close homologue of *A. vinelandii* ferredoxin I, has reduction potentials of −460 and −645 mV for the [3Fe–4S] and [4Fe–4S] clusters, respectively (*318*). The reduction potential of the [3Fe–4S] cluster of *M. smegmatis* ferredoxin was also estimated to be around −435 mV and that for the [4Fe–4S] cluster of *Pseudomonas ovalis* to be lower than −600 mV, these assessments being by EPR (*319*) and NMR (*320*) studies, respectively. Similar values were obtained for the centers of *S. griseus* ferredoxin, measured using EPR (*309*).

It was shown by site-directed mutagenesis and subsequent X-ray crystallographic analysis that the substitution of Cys 20, which was a ligand for the [4Fe–4S] cluster and remote from the other three ligands, by Ala resulted in structural rearrangement that enabled Cys 24, which was in van der Waals contact with the cluster in the authentic ferredoxin, to become an alternative ligand (*321*). The mutated protein showed various spectroscopic properties similar to those of the original protein, implying that both the [4Fe–4S] and [3Fe–4S] clusters were formed, although it showed a considerably different circular dichroism (CD) spectra, reflecting a substantial rearrangement around the [4Fe–4S] cluster. Interestingly, the modified protein was as stable to oxygen as the authentic ferredoxin.

Little is known about the physiological function of *Azotobacter*-type ferredoxins, despite these extensive structural and electrochemical studies. *Streptomyces griseus* ferredoxin is the only one of this group for which a specific function has been proposed (*308*). First, because it is not constitutively expressed but induced along with a cytochrome *P*-450 system by adding soybean flour to the medium, and, second, it can

react with the enzyme *in vitro,* its function may be as an electron donor to cytochrome *P*-450. Genetic studies on *A. vinelandii* ferredoxin I and *R. capsulatus* ferredoxin II are described later (Section IV,D).

C. *Desulfovibrio*-Type [4Fe–4S][3Fe–4S] Ferredoxins

Analysis of aerobically prepared *Desulfovibrio africanus* ferredoxin III by combining direct electrochemical and spectroscopic methods demonstrated that it possessed a [3Fe–4S] cluster with a reduction potential of −140 mV and a [4Fe–4S] cluster of −410 mV (*322*). This ferredoxin has only seven Cys residues; thus the [4Fe–4S] cluster would be formed by Cys 21, 41, 44, and 47, as is usually observed, and the [3Fe–4S] cluster is predicted *in vitro* to be chelated by Cys 11, 17, and 51. The properties of this ferredoxin were the same as that of *D. gigas* ferredoxin II, the tetrameric form (*9*). However, it was also proposed that the cluster might be a [4Fe–4S] form, *in vivo,* with Asp 14 as the fourth ligand, because the reduced [3Fe–4S] cluster could be easily converted to the [4Fe–4S] cluster by the addition of Fe^{2+} ion to give a center with a reduction potential of −400 mV (*323*). This situation is similar to that observed for aconitase (*7*), but the fourth ligand of aconitase was a water molecule instead of the carboxyl side chain (*324*). It should be noted that the residue at position 52, next to Cys 51, is not Pro as usual, but Glu.

Desulfovibrio vulgaris Miyazaki ferredoxin I, which is the major isoform of the two ferredoxins present, was demonstrated to have two redox centers showing distinct behaviors (*325*). It has a sequence highly homologous to that of *D. africanus* ferredoxin III (*326*); 52 residues out of 61 residues are identical, including the sequences of the two Cys clusters with the unique Asp 14 and Glu 52. This ferredoxin was proposed to be the electron carrier in the phosphoroclastic reaction composed of pyruvate dehydrogenase and the hydrogenase–cytochrome c_3 system (*325*).

D. Functional Differences of Distinct Ferredoxins in a Single Bacterium

A number of bacteria have been known to contain multiple ferredoxins that are structurally and phylogenetically distinct. However, it is not known if such multiple ferredoxins are really distinct in physiological function, because almost all of the bacterial ferredoxins are able to transfer electrons in the assays commonly used: $NADP^+$ photoreduction using plant chloroplasts, and some enzyme systems in extracts

from various organisms. Here, we focus on the functional differences of the ferredoxins from *A. vinelandii* and *R. capsulatus;* both organisms possess 2[4Fe–4S] and [4Fe–4S][3Fe–4S] ferredoxins.

Azotobacter vinelandii contains a [4Fe–4S][3Fe–4S] ferredoxin I that is well studied and two other possible 2[4Fe–4S] ferredoxins linked to either *nifB* or *vnfH* (*303, 305*) as described above. The gene for ferredoxin I, *fdxA,* was cloned and the single genomic copy was disrupted by homologous recombination to yield a mutant with essentially the same phenotype as that of the wild type (*327*). Such an event without any significant effect of the mutation was also observed for the flavodoxin gene *nifF* of this bacterium (*328*). The double mutant with both *fdxA* and *nifF* disrupted could grow at only one-third the rate of the wild type in medium replete in ammonium ion, but would grow as fast as the wild type with N_2 as the nitrogen source (*329*). These observations may indicate that the *nifB*-linked ferredoxin fulfills the need for either ferredoxin I or flavodoxin under nitrogen-fixing conditions but cannot compensate for their absence when a high concentration of ammonium ion represses its expression; another ferredoxin gene linked to *vnfH* might not be expressed in the presence of Mo. The disruption mutant of the *nifB*-linked ferredoxin gene, together with an unidentified ORF situated downsteam, will still grow by nitrogen fixation at half the rate of that of the wild type (*303*), probably because either ferredoxin I or flavodoxin can compensate to some extent for its function. If these suppositions are correct, there may be no specific function for *A. vinelandii* [4Fe–4S][3Fe–4S] ferredoxin I.

Possession of two ferredoxins, I and II, by *R. capsulatus* has been observed at the protein level (*330–332*), and the genes *fdxN* and *fdxA* for these ferredoxins have recently been cloned and sequenced (*140, 142, 297*). Northern blotting, in-frame *lacZ* fusion, and Western blotting analyses indicated that *fdxN* is regulated similarly to the *nif* genes (*140, 333, 333a*). Furthermore, the mutants disrupted in *fdxN* scarcely grew on N_2 as the sole nitrogen source, and an *fdxN* nonpolar mutant could be induced to fix N_2 at the wild-type level by reintroducing the gene (*142*). This was good evidence that ferredoxin I was required for nitrogen fixation by the bacterium. Another ferredoxin gene was identified during studies on genes downstream of *nifENX* and this encoded a product of 101 residues with two Cys clusters of the typical -C-X-X-C-X-X-C-X-X-X-C-P- sequence. However, it had a longer insertion sequence between the two clusters of 42 residues compared to the usual 17 residues found in clostridial ferredoxins (*334*). The product is probably a 2[4Fe–4S] ferredoxin. Interestingly, it was pointed out that the insertion length was identical to that found in *nifJ,* which encodes

the pyruvate–flavodoxin oxidoreductase of *Klebsiella pneumoniae* (*335*). Disruption of this gene together with the partly overlapping *nifQ* rendered the bacterium unable to grow on N_2, but the addition of a high concentration of Mo (1 m*M*) to the medium overcame this disability (*334*). Hence, the phenotype could be attributed to *nifQ* disruption. The fourth ferredoxin gene, *fdxC*, which encodes a protein homologous to plant [2Fe–2S] ferredoxins, was found just upstream of *fdxN* (*142*) and was shown also to be required to some extent for nitrogen fixation (Section II,C,2).

Therefore, three of the ferredoxin genes belonging to the *nif* gene family have been accounted for. The remaining [4Fe–4S][3Fe–4S] ferredoxin II encoded by *fdxA* was expressed constitutively under various growth conditions: photosynthesis, respiration, anaerobic respiration, or nitrogenase repressed or derepressed, as analyzed by transcriptional analysis using in-frame *lacZ* fusion (*333a, b*). In addition, *fdxA* cannot to date be disrupted, even by screening under the conditions that derepress the expression of the other three ferredoxin genes (*142*). These observations indicate that *R. capsulatus* [4Fe–4S][3Fe–4S] ferredoxin II has some specialized physiological function indispensable for the bacterium, unlike the case of the analogous protein from *A. vinelandii*. The biochemical significance in structure–function relationships in these ferredoxins remains to be elucidated.

Some nitrogen-fixing bacteria possess multiple ferredoxins apparently related to nitrogen fixation. *Rhizobium meliloti fdxN* and *fixX*, which encode possible 2[4Fe–4S] and [4Fe–4S] ferredoxins, respectively, appear to be essential for nitrogen fixation (*224, 225, 301*). The functional significance of an *Anabaena* PCC7120 ferredoxin, the *fdxN* product, remains to be elucidated, but is of great interest because of its uniqueness in bacterial cellular and genomic differentiation (*300*) and because of its relationship to the *fdxH*-encoded product, the heterocystous [2Fe–2S] ferredoxin (Section II,A).

E. Other Ferredoxins

Clostridium perfringens ferredoxin was previously reported to have a single [4Fe–4S] cluster (*336*), but its sequence was highly homologous with that of *C. pasteurianum* ferredoxin (*337*), suggesting it might contain 2[4Fe–4S] clusters. It donates electrons to nitrate reductase (*338*).

Butyribacterium methylotrophicum, a methylotrophic heteroacetogen, has a ferredoxin whose physicochemical properties and sequence

are very similar to those of *C. pasteurianum* ferredoxin (*339, 340*). It interacts with hydrogenase but only poorly with the CO dehydrogenase of the bacterium. Ferredoxin and rubredoxin phylogenetic trees constructed for seven bacteria showed essentially the same topology (*340*).

Methanococcus thermolithotrophicum, a thermophilic methanogen, produces a thermostable ferredoxin that will withstand 88°C for 1 hr, but that is oxygen unstable (*236*). It has about 60 residues, including eight cysteines, and is thought to function as an electron acceptor for the CO dehydrogenase complex.

F. F_A/F_B Proteins in Photosystem I Complexes

As mentioned in Section III,F,1, the PS I complex has three Fe–S centers, X, A, and B. An 8- to 9-kDa polypeptide associated with the complex was suggested to be an Fe–S protein (*341–343*) and accepts electrons from the Fe–S center X and donates them to the [2Fe–2S] ferredoxin. The sequence analysis of the 9-kDa polypeptide proved it to be the apoprotein that carries centers A and B (*344–346*), i.e., F_A/F_B protein. Spinach protein was partially purified (*347*) and reconstituted with the PS I complex from which F_A/F_B protein was depleted (*348*). Thus, the 9-kDa F_A/F_B protein encoded by *psaC* (or *frxA* of liverwort) was identified to be an Fe–S protein with centers A and B. Spinach F_A/F_B protein isolated with partially degraded Fe–S clusters showed an amino acid sequence similar to those of ferredoxins of the clostridial 2[4Fe–4S] type (*349*) and the presence of two distinct Fe–S clusters was indicated by EPR spectroscopy (*350*). When the F_A/F_B protein was exposed to air, the cluster was rapidly destroyed (*351*), in contrast to most of the clostridial 2[4Fe–4S] ferredoxins. Results of treatments with alkali, chaotropic ions, trypsin, and cross-linking reagents indicated that the F_A/F_B protein was located peripherally on the stromal side of the thylakoid membranes, but was embedded under two other subunits situated closely to it (*352*). The protein was finally obtained from spinach leaves in a native state by isolation under anaerobic conditions (*351*). The iron and inorganic sulfur contents and the spectroscopic properties of the protein are nearly identical with those of the 2[4Fe–4S] ferredoxins of clostridial type. The reduction potentials of the two clusters were estimated to be -470 and -560 mV, respectively, although in complex I they were measured at -550 and -590 mV (*353*).

Amino acid sequences of several F_A/F_B proteins are known (*272, 273, 349, 354–358*), two of which (*349, 354*) were found by protein

sequencing. They are highly conserved with about 79% homology and are most closely related to *Chromatium* 2[4Fe–4S] ferredoxin (*349*). The cysteine residues at positions 10, 13, 16, 20, 47, 50, 53, and 57 are probably responsible for chelating the two [4Fe–4S] clusters in a manner similar to those of clostridial ferredoxins. Unlike the usual ferredoxins, these F_A/F_B proteins are weakly basic and rather hydrophilic in spite of their location as a component of a membrane protein complex (*349*). They are slightly larger than clostridial ferredoxins, with an insertion in the middle portion of the molecule and an extension at the C terminus. Figure 11 compares the sequence of spinach F_A/F_B protein with those of several bacterial ferredoxins. A three-dimensional structure was simulated (*349*) on the basis of the tertiary structure of *P. aerogenes* 2[4Fe–4S] ferredoxin (*218*), as given in Fig. 7.

G. Ferredoxin-Like Proteins Encoded by the Chloroplast and Cyanobacterial Genes: *frxB*, *orf167*, and *orf178*

Chloroplast DNAs of liverwort (*272*), tobacco (*273*), and rice (*359*) contained *frxB*, *orf167*, and *orf178*, respectively, which could encode putative ferredoxin sequences containing 2[4Fe–4S] clusters typical of clostridial 2[4Fe–4S] ferredoxins; their function is unknown. Corre-

```
(a) SHS-VKIYDTCIGCTQCVRACPTDVLEMIPWDGCKAK
(b)   A-YKIADSCVSCGACASECPVNAI--------SQG
(c)   ALY-ITEECTVCGACEPECPVTAI--------SAG
(d)   ALM-ITDQCINCNVCQPECPNGAI--------SQG

(a) QIASAPRTEDCVGCKRCESACPTDFLSVRVYLWHETT
(b) DSIFVIDADTCIDCGNCANVCPVGAPVQE
(c) DDIYVIDANTCNECAACVAVCPAECIVQG
                 [GLDEQ-]
(d) DETYVIEPSLCTECVQCVEVCPVDCIIKDPS--HEET
                 [GHYETS]

(a) ----------RSMGLGY
(d) EDELRAKYERITGEG
```

FIG. 11. Comparison of the sequences of spinach 9-kDa F_A/F_B protein and bacterial 2[4Fe–4S] ferredoxins. Gaps are inserted to maximize homology in the alignment (*349*). Cysteine residues suggested to chelate iron atoms are italicized. (a) Spinach F_A/F_B protein (*349*), (b) *C. pasteurianum* ferredoxin (*9*), (c) *C. limicola* ferredoxin I (*291*), and (d) *C. vinosum* ferredoxin (*293*).

sponding genes were also identified in wheat (*360*) and *P. boryanum* (*361*). Their residue numbers ranged from 167 to 194 and they show substantial homology to each other, but are greatly divergent from bacterial ferredoxins (Fig. 12).

For *Chlamydomonas*, spinach, and tobacco (*363, 364*) the gene product was detected as an Fe–S protein (*363*) and located as a membrane-bound component on the peripheral portion of the thylakoid membranes. The *frxB* gene is in a gene cluster encoding four proteins homologous to mitochondrial complex I components. Several nuclear-encoded subunits of the mitochondrial complex I also have proteins homologous to those encoded by ORFs in chloroplast genomes, e.g., the 23-kDa subunit of bovine complex I is homologous to the *frxB* gene product (*365–367*), though to a lesser extent than seen in comparing *frxB* genes from chloroplasts. Another *frxB* homologue was found in the *E. coli hyc* operon encoding formate hydrogenlyase (*368*). These observations led to the suggestion that the *frxB*-encoded protein functions as a redox component of an NAD(P)H : plastoquinone reductase. A regulatory function in chloroplast DNA replication was also suggested (*363*). However, these are indirect bits of evidence and the real functional role of this protein in the thylakoid membranes is still unclear (*361*).

V. Polyferredoxins

Methanobacterium thermoautotrophicum, an archaebacterium, reduces CO_2 with H_2 to give CH_4, with hydrogenases being indispensable

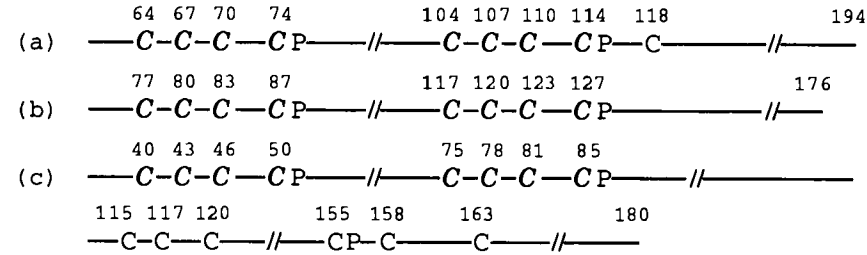


FIG. 12. Comparison of the sequences of *frxB*-encoded proteins and homologues. The positions of cysteine and proline residues are given. Cysteine residues suggested to chelate iron atoms are italicized. The *frxB*-encoded proteins of (a) *Plectonema boryanum* (194 residues) (*361*), liverwort (183 residues) (*284*), tobacco (167 residues) (*362*), rice (178 residues) (*359*), and wheat (176 residues) (*360*); (b) bovine mitochondrial complex I, 23-kDa subunit (*367*); and (c) *E. coli* formate hydrogenlyase *orf6* gene product (*368*).

enzymes for the reaction (*369*). The genes, *mvhDAG,* encode subunits of the methyl viologen-reducing hydrogenase of this bacterium. The organization and sequences of the genes indicated that they shared a common ancestor with eubacterial hydrogenase genes. The putative *mvhB* gene product contained six tandemly repeated domains corresponding to those of bacterial 2[4Fe–4S] ferredoxins, suggesting it was a polyferredoxin that contained 12[4Fe–4S] clusters. Another polyferredoxin sequence was also deduced from the DNA sequence of the *mvhB* gene of *Methanothermus fervidus,* which suggested the protein would have domains similar to the 2[4Fe–4S] ferredoxins and 64% identity to the *M. thermoautotrophicum* protein (*370*).

VI. Conclusions

Many ferredoxins and related (ferredoxin-like) proteins of different types have now been isolated from various organisms, and some of them have been well characterized in terms not only of physiological functions but also of chemical and physical aspects. With increasing emphasis on gene structural studies, a rapid accumulation of data on new ferredoxins and related proteins, in particular their primary structures, has resulted. The biosynthetic and regulatory mechanisms of these proteins have been the subject of recent interest. In particular, many iso-forms of ferredoxins have been detected in individual organisms ranging from higher plants to bacteria. The likelihood of distinct functions for the iso-forms in some organisms has been discussed, with the recent introduction of new approaches in genetic manipulations enabling more informed speculation on this. Table I gives some examples of iso-forms of ferredoxins and related proteins in some photosynthetic organisms. Nearly 20 years ago several investigators reported the presence of iso-forms of ferredoxins in various photosynthetic and nonphotosynthetic bacteria and oxygenic photosynthetic organisms (see Ref. *371* and references therein), but the significance of this multiplicity was far from clear. The first clear evidence of a distinct function for an iso-ferredoxin was the isolation of a ferredoxin from the heterocysts of the cyanobacterium, *Anabaena variabilis.* Only the heterocystous ferredoxin donated electrons to nitrogenase and the vegetative cell ferredoxin was ineffective in this reduction. Another ferredoxin in the heterocysts corresponded to the vegetative cell type and was assumed to function in the adapted photosynthetic system of this specialized cell to supply energy for nitrogen fixation. The [2Fe–2S] ferredoxins found in plant leaves and other oxygenic photosynthetic organisms function

TABLE I

NUMBER OF FERREDOXINS AND FERREDOXIN-LIKE PROTEINS IN PHOTOSYNTHETIC ORGANISMS

Source	Ferredoxin and related protein			HiPIP
	2[4Fe–4S]	[4Fe–4S] · [3Fe–4S]	[2Fe–2S]	[4Fe–4S]
Spinach				
Leaves	2[a,b]	—	2	—
Roots	—	—	1	—
Anabaena PCC7120				
Vegetative cells	(2)[c]	—	1	—
Heterocysts	1 + (2)[c]	—	2	—
Chlorobium limicola	2	—	—	—
Chromatium vinosum	1	—	—	1
Rhodobacter capsulatus	2	1	1	—

[a] The reaction center F_A/F_B protein encoded by the *frxA* (liverwort) gene.

[b] The membrane-bound protein encoded by the *frxB* (liverwort) gene.

[c] The parentheses indicate the probable presence of proteins comparable to those indicated in footnotes *a* and *b*.

as the electron donors to $NADP^+$, but iso-forms of these have also been found in nonphotosynthetic tissues such as white roots. Tissue-specific ferredoxins have also been found in C_4 plants such as maize. Their functions other than in photosynthesis have been the subject of speculation and answers to this question can be anticipated in near future. Another differentiation of the function of iso-ferredoxins was first demonstrated in the photosynthetic purple nonsulfur bacteria, exemplified by *R. capsulatus*. They have at least four ferredoxins of three different types as listed in Table I. Gene inactivation experiments have revealed that two of them, the 2[4Fe–4S] and [2Fe–2S] ferredoxins, function in nitrogen-fixing systems and the [4Fe–4S][3Fe–4S] ferredoxin functions in some as-yet unidentified system that nevertheless has a critical role for the survival of this bacterium. *Chlorobium limicola* was shown to have two ferredoxins with sequences that differed at only a relatively few positions, and these may not have distinct functions. There is no indication whether these bacterial ferredoxins have a specific location in the cell or are associated with particular enzyme or protein complexes. Furthermore, it was surprising to locate in chloroplasts ferredoxin-like proteins very similar to the bacterial 2[4Fe–4S] ferredoxin. One of these functioned as the reaction center protein, F_A/F_B, in pho-

tosystem I of oxygenic photosynthesizers and the other was also found in photosynthetic organisms in a membrane-bound form, though at the moment there is no evidence of its function. Another Fe–S protein encoded by liverwort chloroplast DNA, but not by higher plant chloroplast DNA, is similar to the Fe protein of nitrogenase in prokaryotes. This product of the *frxC* gene has been shown to function in chlorophyll biosynthesis, and not in nitrogen fixation, of which the organism is incapable.

We have briefly reviewed in this article a number of aspects of the structure and functional diversity of ferredoxins and their homologues, but have not included the mechanism of Fe–S cluster formation *in vivo,* which is beyond the scope of the present review. However, recent studies conducted with spinach chloroplasts (*372–374*) are very interesting in demonstrating that ATP and NADPH are required to form the Fe–S cluster of spinach ferredoxin, when cysteine is used as the sulfur donor. This study is still in progress and we have no idea if the same mechanism as suggested for the spinach chloroplast system can be true also for other Fe–S proteins. There is no doubt that other important and interesting ferredoxins and related proteins will continue to be found and their physical and chemical characterizations, as well as studies of their physiological roles, will further broaden our insight into this important and diverse group of proteins.

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