

Clostridial Iron-Sulphur Proteins

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Abstract

Iron-sulfur proteins are ubiquitous catalysts of a wide range of biological reactions, and are particularly abundant in clostridia which lack the ability to synthesize hemes. The development of research on these metalloproteins has therefore been strongly associated with biochemical investigations of clostridial metabolism. Major breakthroughs in the field, from the first isolation of an iron-sulfur protein in 1962, to the recent determination of an Fe-hydrogenase structure, have been made with clostridia. These data, as well as others obtained through studies on clostridia, are transferable to many other bioenergetic machineries, due to the strong phylogenetic conservation of some important components. For instance, clear homologies exist between constituents of the anaerobic electron transfer chains in clostridia and aerobic respiratory chains. The contribution of iron-sulfur proteins to the biotechnological and medical significance of clostridia is also discussed. Structural and functional genomics are expected to bring forth a wealth of novel data on clostridia and iron-sulfur proteins.

Introduction

Iron-sulfur (Fe-S) proteins contain active sites consisting of variable numbers (one to eight) of inorganic sulfide (S²⁻) and iron atoms bound to the polypeptide chain by cysteinyl sulfur atoms (Johnson, 1994 ; Beinert *et al.*, 1997). In some rare cases histidine ligation has been observed (Moulis *et al.* 1996 ; see section on hydrogenase below). The fact that Fe-S proteins are ubiquitous catalysts and regulators in living cells may be related to a possible role of iron-sulfur chemistry in the origin of life (Huber and Wächtershäuser, 1998; Russell and Hall, 1997). These proteins are particularly abundant and diverse in clostridia which lack the heme synthesis machinery. For circumstantial reasons, in particular early studies on the biochemistry of nitrogen fixation (Carnahan *et al.*, 1960; Mortenson *et al.*, 1962; Hardy *et al.*, 1965), *Clostridium pasteurianum* is, among clostridia, the best source of well characterized Fe-S proteins. However, most of the data can be extrapolated to other clostridia, including the pathogens and those of biotechnological significance.

Abbreviations

Fe-S: iron sulfur; H₂ase: hydrogenase; PFOR: pyruvate-ferredoxin oxidoreductase.

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The aim of this review is twofold: first, to point out the importance of Fe-S proteins in clostridial metabolism, and second, to show that clostridia, as efficient and versatile synthesizers of Fe-S proteins, provide a cornucopia of information on the structure and function of these proteins in all kinds of organisms.

Rubredoxins

As the simplest of all Fe-S proteins, rubredoxins from anaerobic bacteria comprise 45 to 54 residues, and their active site consists of a single iron coordinated to four cysteinyl sulfurs. Rubredoxin-encoding genes have been found in *C. pasteurianum* (Mathieu *et al.*, 1992), *C. beijerinckii* (Wilkinson and Young, 1995), *C. perfringens* (Katayama *et al.*, 1995), *C. acetobutylicum* (Cornillot *et al.*, 1997), and *C. butyricum* (Gérard *et al.*, 1999). At least four primary structures (*C. pasteurianum*, *C. perfringens*, *C. sticklandii*, and *C. thermosaccharolyticum*) of clostridial rubredoxins have been determined either by protein or by DNA sequencing (reviewed in Mathieu *et al.*, 1992).

The monocistronic *C. pasteurianum* rubredoxin gene has been cloned, sequenced, and expressed in *E. coli* (Mathieu *et al.*, 1992; Mathieu and Meyer, 1993). Both the native-like Fe-containing and a Zn-substituted forms of the protein have thus been isolated and structurally characterized (Dauter *et al.*, 1996). The heterologous expression system has allowed isotopic labeling of *C. pasteurianum* rubredoxin and assignment of previously unobserved NMR resonances of the cysteine ligands of the iron (Xia *et al.*, 1995). Many mutated forms of rubredoxin have been prepared with the aims of understanding the stability (Eidsness *et al.*, 1997) and the electron transfer properties of this protein (Kümmerle *et al.*, 1997) or assembling novel metal sites (Meyer *et al.*, 1995; 1997; Xiao *et al.*, 1998).

The yet unknown function of rubredoxins in clostridia might be analogous to the one hypothesized in sulfate reducing bacteria, namely a possible involvement in the protection against oxygen (Voordouw and Voordouw, 1998).

[2Fe-2S] Ferredoxin

This protein (Hardy *et al.*, 1965) has been sequenced and characterized by various spectroscopic techniques (reviewed in Meyer *et al.*, 1994). It is a dimer of a 102-residue polypeptide chain containing one [2Fe-2S] cluster per subunit. The encoding gene is monocistronic (Meyer, 1993) and has been expressed in *E. coli* (Fujinaga and Meyer, 1993). Unique structural features of this protein have been uncovered by site-directed mutagenesis (Meyer *et al.*, 1994 ; Golinelli *et al.*, 1996 ; 1998). Molecular variants with cysteine ligands of the Fe-S cluster substituted with serine (Fujinaga *et al.*, 1993 ; Meyer *et al.*, 1994) have allowed the discovery of novel properties of [2Fe-2S] active sites (Crouse *et al.*, 1995 ; Achim *et al.*, 1996 ; 1999).

The *C. pasteurianum* [2Fe-2S] protein is totally unrelated to plant-type [2Fe-2S] ferredoxins, in particular because of its unusual cysteine residue pattern, and has long remained unique (Meyer, 1988). It appears to have no counterparts in other members of the genus *Clostridium* (Wilkinson and Young, 1995; Katayama *et al.*, 1995; Cornillot *et al.*, 1997). Subunits or domains homologous to the *C. pasteurianum* [2Fe-2S] protein have now been evidenced in numerous redox enzymes and complexes, e.g. hydrogenases (Chatelet *et al.*, 1999), or subunit NuoE of Complex I in respiratory chains (Ohnishi, 1998). Furthermore, genes encoding two very similar proteins from *Azotobacter vinelandii* (Chatelet and Meyer, 1999) and *Aquifex aeolicus* (Chatelet *et al.*, 1999) have been expressed in *E. coli*.

The *C. pasteurianum* [2Fe-2S] protein is synthesized in larger amounts under nitrogen fixing conditions (Hardy *et al.*, 1965), and the encoding gene, though apparently monocistronic, is surrounded by *nif*-related genes (Meyer, 1993). These hints have recently been substantiated by the observation that the [2Fe-2S] protein interacts specifically with the nitrogenase MoFe protein (Golinelli *et al.*, 1997). The functional meaning of this interaction nevertheless remains to be elucidated.

2[4Fe-4S] Ferredoxins

The 2[4Fe-4S] ferredoxin from *C. pasteurianum* was the first Fe-S protein ever isolated (Mortenson *et al.*, 1962) and was soon found to shuttle electrons between a number of redox enzymes. For instance, it can be reduced by pyruvate-ferredoxin oxidoreductase (PFOR) and then reoxidized by hydrogenase (H₂ase, see below) which disposes of the cellular excess of reducing equivalents by producing dihydrogen (Lovenberg *et al.*, 1963). Several clostridial 2[4Fe-4S] ferredoxins have been characterized (Lovenberg *et al.*, 1963) and sequenced (compiled in Moulis and Davasse, 1995). The crystal structure of the ferredoxin from *C. acidurici* (formerly *C. acidurici* and *C. acidu-urici*, Cato and Stackebrandt, 1989) has been determined to the very high resolution of 0.94 Å, allowing a detailed analysis of the geometry of the metal sites (Dauter *et al.*, 1997). The gene encoding this protein is monocistronic and a single copy appears to be present in the genome (Graves *et al.*, 1985). Expression in *E. coli* of the natural gene (Baur *et al.*, 1990) and of a synthetic gene optimized for expression in *E. coli* (Davasse and Moulis, 1992) has been achieved. The latter has been implemented in an extensive site-directed mutagenesis program aimed at analyzing the features contributing to the stability and structure of the protein (Quinkal *et al.*, 1994), its intramolecular electron transfer properties (Kyritsis *et al.*, 1997), and its interactions with H₂ase and PFOR (Moulis and Davasse, 1995). This important protein, which is most certainly present in all clostridia, can be used as an iron supply by the cells when conditions of iron starvation are set up (Schönheit *et al.*, 1979), and is replaced by flavodoxin under these conditions (Knight and Hardy, 1966).

Many redox enzymes or complexes in anaerobic (see below the sections on PFOR and H₂ase), photosynthetic (subunit PsaC of photosystem I; Schubert *et al.*, 1997), and aerobic organisms (subunit NuoI of Complex I; Ohnishi, 1998), contain subunits or domains homologous to clostridial 2[4Fe-4S] ferredoxins.

Pyruvate-Ferredoxin Oxidoreductase (PFOR)

This enzyme oxidizes pyruvate by forming CO₂ and acetylCoA and reducing the 2[4Fe-4S] ferredoxin. Clostridial PFORs are homodimers of ca. 240 kDa which have been reported to contain one or two [4Fe-4S] clusters per subunit (Moulis *et al.*, 1996, and references therein). Recent sequence data (J. Meyer, Genbank entries AF064035 and AF064550; F. Rodriguez *et al.*, Genbank entries Y17726 and 17727; Horner *et al.*, Genbank entry AF132673) indicate that *C. pasteurianum* contains at least three putative PFOR-encoding genes. One of these (Genbank entry AF064035) has been identified on the basis of partial protein sequence data (J. Meyer, unpublished) as encoding the biochemically characterized protein (Moulis *et al.*, 1996). The two additional putative genes might encode enzymes specific of ketoacids other than pyruvate. Alternatively, one of them might be the counterpart of the *nifJ* gene found in other nitrogen-fixing bacteria (Peters *et al.*, 1995). Whatever the functions of these genes, all of the three translated sequences are very similar to the sequence of the PFOR from *Desulfovibrio africanus*, of which the crystal structure has recently been determined (Chabrière *et al.*, 1999). The latter structure therefore provides a good model for clostridial PFOR. The size and dimeric structure are confirmed, but each subunit contains three [4Fe-4S] clusters in addition to the thiamine pyrophosphate active site. It is worth noticing that the two [4Fe-4S] clusters that are closest to the protein surface are accommodated in a protein fold homologous to 2[4Fe-4S] ferredoxins (Chabrière *et al.*, 1999).

Hydrogenase

Clostridial hydrogenases (H₂ase), by reducing protons to dihydrogen, perform an essential cellular function in disposing of the excess reducing equivalents. They belong to the Fe-only class of these enzymes (Adams, 1990). Their high catalytic activity had soon caught the attention of those involved in the development of biohydrogen technologies (Rao *et al.*, 1976). An H₂ase from *C. pasteurianum* has been purified (Chen and Mortenson, 1974) and characterized by biochemical and spectroscopic methods (Adams, 1990). Later on, a second enzyme (H₂ase II) was isolated and reported to be considerably more active in the reverse, i.e. dihydrogen uptake, than in the forward reaction (Adams, 1990). It was reported to be somewhat smaller than H₂ase I (56 versus 64 kDa), but to contain a similar hydrogen-activating site. Whereas H₂ase I has subsequently been investigated in considerable detail (see below), H₂ase II has not been heard of since. However, the currently sequenced genome of *C. acetobutylicum* ATCC 824 appears to contain, in addition to the previously sequenced H₂ase gene (Gorwa *et al.*, 1996), a putative gene (orf 3023, see <http://pedant.mips.biochem.mpg.de>) that might encode a counterpart of H₂ase II from *C. pasteurianum*.

The H₂ase I-encoding gene from *C. pasteurianum* has been cloned using oligonucleotide probes derived from peptide sequence data (Meyer and Gagnon, 1991; Meyer, 1995). Other H₂ase-encoding genes, monocistronic like the one from *C. pasteurianum*, have subsequently been cloned in *C. acetobutylicum* P262 (Santangelo *et al.*, 1995) and ATCC 824 (Gorwa *et al.*, 1996). The strikingly modular

sequence of the *C. pasteurianum* H₂ase gene suggested that the protein was composed of well defined domains. Indeed, expression in *E. coli* of gene fragments lead to the purification of an N-terminal 76-residue domain (Atta *et al.* 1998) and the demonstration by 2D-NMR that it folds like [2Fe-2S] plant-type ferredoxins (Kümmerle *et al.*, 1999). The crystal structure of the whole enzyme (Peters *et al.*, 1998) confirmed its modular structure: the plant ferredoxin-like domain is followed by a novel domain containing a [4Fe-4S] cluster with three cysteine and one histidine ligand, then by a domain homologous to clostridial 2[4Fe-4S] ferredoxins, and finally by a large (more than half of the sequence) C-terminal domain containing the hydrogen-activating site. The latter is a novel six-iron cluster consisting of a [4Fe-4S] unit connected by a bridging cysteinyl sulfur to a CO- and CN-ligated two-iron unit (Peters *et al.*, 1998; Nicolet *et al.*, 1999). It is worth mentioning that taken together, the N-terminal domains (ca. 220 residues) preceding the active site domain are homologous to the N-terminus of the NuoG subunit of Complex I (Atta *et al.*, 1998; Kümmerle *et al.*, 1999).

The determination of crystal structures of Fe-H₂ases (Peters *et al.*, 1998; Nicolet *et al.*, 1999), together with the previously determined NiFe-H₂ase structure (Volbeda *et al.*, 1995), are important steps towards understanding the mechanism of hydrogen activation. The way would now be paved for further investigations of Fe-H₂ases by site-directed mutagenesis, but the complex hydrogen-activating metal site is not assembled in *E. coli* (Voordouw *et al.*, 1987), and the genes committed to this task have not yet been identified in any organism. In the present state of the art, the enzyme from sulfate reducers (Nicolet *et al.*, 1999) would appear to be more tractable than the clostridial one.

Nitrogenase

The ATP-dependent reduction of dinitrogen to ammonia is a biogeochemically important reaction carried out in a variety of prokaryotes by the enzyme nitrogenase. *C. pasteurianum* has been a prominent microorganism in the investigation of nitrogen fixation (Carnahan *et al.*, 1960). Crystal structures of both protein components of *C. pasteurianum* nitrogenase are available. The homodimeric ($\gamma_2=60$ kDa) Fe protein contains a single [4Fe-4S] cluster held between the two subunits (Schlessman *et al.*, 1998). Each $\alpha\beta$ pair of the heterotetrameric ($\alpha_2\beta_2=220$ kDa) MoFe protein contains one eight-iron cluster and one FeMo cofactor. The latter is composed of one Mo, seven Fe, nine sulfide atoms, and one homocitrate molecule (Kim *et al.*, 1993). Electron transfer from the Fe protein to the MoFe protein is coupled to ATP hydrolysis and is rate limiting in the overall reaction. Substrate reduction takes place on the FeMo cofactor (Peters *et al.*, 1995).

Investigations on *Klebsiella pneumoniae* and *A. vinelandii* have shown that over twenty *nif* genes are involved in the biosynthesis and regulation of nitrogenase (Peters *et al.*, 1995). The synthesis of alternative nitrogenases containing vanadium instead of molybdenum (*vnf* genes) or iron only (*anf* genes) is induced in some organisms under conditions of molybdenum, or molybdenum and vanadium starvation, respectively (Bishop and Joerger, 1990). Whereas some bacteria contain all three nitrogenase systems (e.g. *A. vinelandii*), *C. pasteurianum* appears to contain only the *nif* and *anf*

systems (Johnson *et al.*, 1993; Zinoni *et al.*, 1993; Loveless and Bishop, 1999). Although a full inventory of the *C. pasteurianum nif* and *anf* genes remains to be carried out, it already appears that the *nif* genes are split into several operons (Johnson *et al.*, 1993) rather than clustered in a single one as in *K. pneumoniae*.

Though *C. pasteurianum* is by far the best investigated member of the genus with respect to N₂ fixation, some other clostridia appear to be nitrogen fixers (Rosenblum and Wilson, 1949; Kanamori *et al.*, 1989, and references therein). However, biochemical and genetic confirmation of these observations remain to be brought forth.

Other Iron-Sulfur Proteins

Among the other clostridial Fe-S proteins, the CO dehydrogenase/acetyl-CoA synthase from *C. thermoacetatum* has been one of the most thoroughly characterized, although its three-dimensional structure is still unknown. Biochemical and spectroscopic investigations have revealed the following (Ragsdale and Kumar, 1996). The protein is an $\alpha_2\beta_2$ tetramer. The acetyl-CoA synthase activity resides in the α subunit (78 kDa) which contains a [4Fe-4S] cluster bridged to a nickel atom. The CO-dehydrogenase activity resides in the β subunit (71 kDa) which contains a classical [4Fe-4S] cluster and a [4Fe-4S] cluster bridged to a Ni atom. The [4Fe-4S]-Ni sites in the α and β subunits display significant spectroscopic differences.

The 4-hydroxybutyryl-CoA dehydratase from *Clostridium aminobutyricum* (Muh *et al.*, 1996) is a member of a series of enzymes catalyzing the elimination of water from various hydroxyacyl derivatives. It is an homotetramer in which each of the 56 kDa subunits contains one FAD and one [4Fe-4S] cluster. FAD might be involved in a transient one electron oxidation of the substrate to activate the β -CH bond, whereas the Fe-S cluster, as in many dehydratases/isomerases, would work as a Lewis acid facilitating the leaving of the hydroxyl group.

Lysine 2,3-aminomutase from *Clostridium subterminale* SB4 catalyzes the interconversion of L-lysine and L- β -lysine (Lieder *et al.*, 1998). It is composed of six 47 kDa subunits containing one [4Fe-4S] cluster each, and requires pyridoxal-5'-phosphate and S-adenosylmethionine for activity. The Fe-S cluster presumably serves as a reductant of S-adenosylmethionine.

Pyruvate formate lyase, which catalyzes the formation of formate and acetyl-CoA from pyruvate and CoA, is central in the anaerobic metabolism of *E. coli*, and is also present in anaerobes such as *C. pasteurianum* (Weidner and Sawers, 1996). It belongs to a family of glycol-radical enzymes in which radical generation is achieved by an Fe-S activating enzyme. The active form of the latter protein appears to contain a [4Fe-4S] cluster, but reversible conversions into a [2Fe-2S] cluster have been reported (Sawers, 1999).

Several clostridial species contain formate dehydrogenases or carboxylic acid reductases (CAR) which are Mo or W enzymes containing Fe-S sites as well, most often [4Fe-4S] clusters (Johnson *et al.*, 1996). The CAR are related to the hyperthermophilic aldehyde oxidoreductases, and are often found in thermophilic acetogenic clostridia (*C. thermoacetatum* and *C. formicoaceticum*). The genes encoding the two subunits

of the W- and selenocysteine-containing formate dehydrogenase from *C. thermoaceticum* have been sequenced (Li *et al.*, Genbank accession U73807).

The nicotinic acid hydroxylase from *C. barkeri* is composed of four different subunits, and contains Mo, FAD, selenium, and two [2Fe-2S] clusters (Gladyshev *et al.*, 1996). It bears some similarity to the xanthine dehydrogenase from *C. acidurici* (Wagner *et al.*, 1984).

Clostridium thermoaceticum contains enzymes catalyzing the oxidation or reduction of NAD(P)(H) by artificial redox agents. The function of these proteins is unknown, but rubredoxins might be possible partners *in vivo*. These oligomeric proteins contain Fe-S clusters of which the structure and stoichiometry remains to be determined (Bayer *et al.*, 1996).

Fe-S Proteins and Pathogenicity

While Fe-S proteins are very unlikely to be directly involved in clostridial pathogenesis (Rood *et al.*, 1997), some of these proteins have important roles in the prevention and cure of clostridial infections.

Nitrite is added to food, in particular meat products, as a preservative against toxin-forming bacteria such as *C. botulinum*. The bactericidal agents are most probably derivatives of nitrite rather than nitrite itself. These derivatives can be formed during food processing, but also by the pathogenic cells themselves (Cammack *et al.*, 1999). A particularly reactive derivative of nitrite is nitric oxide, which is produced by non-specific reduction of nitrite at low potential, and which can then inactivate Fe-S proteins, in particular the least stable ones (Meyer, 1981). Possible targets would include H₂ase and PFOR.

The specific antibiotic effects of metronidazole and other 5-nitroimidazoles against anaerobes appear to result from their reduction to highly reactive derivatives (Church and Laishley, 1995). This activation requires the low redox potentials occurring in anaerobic cells, and it is most likely carried out by Fe-S proteins. H₂ase (Church and Laishley, 1995), but also ferredoxin and PFOR are likely catalysts of such reactions.

Fe-S Proteins and Solvent Production

The metabolic pathways leading from acetylCoA to ethanol, acetone and butanol are not known to involve Fe-S enzymes. However, some of these proteins, as shown above, produce and redistribute reducing equivalents which can then be used in solventogenic reactions (Mitchell, 1998). PFOR, an essential agent in the carbon flow, also reduces ferredoxin, which can in turn be used to reduce NAD(P). The reducing equivalents transported by ferredoxin can thus be fed into the solvent producing pathways. Alternatively, these electrons can be delivered to H₂ase and disposed of in the form of dihydrogen. Finding ways of cancelling or controlling H₂ase activity at will during the growth cycle is therefore a challenge in solvent production technology: the reducing power dissipated by H₂ase could instead be used for butanol and acetone production (Girbal and Soucaille, 1998). More generally, the balanced distribution of reducing equivalents among the pathways of interest by various enzymes (NAD(P)-ferredoxin or NAD(P)-rubredoxin oxidoreductases) is a field of which the enzymology would deserve increased attention.

Conclusions

Fe-S proteins have been known to be essential catalysts in clostridial cells for several decades, and these bacteria, in particular *C. pasteurianum*, have been instrumental in the development of research on this ubiquitous class of proteins. This trend has been confirmed recently by breakthroughs based at least in part on clostridial proteins. The X-ray structures of Fe-H₂ases (Peters *et al.*, 1998; Nicolet *et al.*, 1999) and PFOR (Chabrière *et al.*, 1999) have unveiled not only novel protein and Fe-S active site structures, but also the detailed paths of electrons in the crucial metabolic pathway linking pyruvate and dihydrogen. Similar achievements concerning other chains of reactions should be expected from the current development of structural and functional genomics.

Fe-S proteins are excellent markers of the protracted evolutionary process leading from the anaerobic to the aerobic lifestyle. Indeed, the bioenergetic machineries of aerobic organisms include components, and in particular Fe-S proteins, that can be traced back to anaerobes. For instance, most, if not all, Fe-S-containing subunits of Complex I of aerobic respiratory chains (Ohnishi, 1998) are related to proteins or protein domains present in clostridia. The homologous pairs include NuoI and the 2[4Fe-4S] ferredoxin, NuoE and the *C. pasteurianum* [2Fe-2S] ferredoxin, and the N-termini of NuoG and H₂ase. These relationships encompass at least six of the eight or nine Fe-S clusters present in Complex I. While high resolution structural data are unavailable for Complex I, they have been obtained for the 2[4Fe-4S] ferredoxin (Dauter *et al.*, 1997), for H₂ase (Peters *et al.*, 1998), and are expected for the [2Fe-2S] ferredoxin. Thus, the structure of the protein environment of most Fe-S clusters of Complex I can be derived from structural data on clostridial proteins. These structure comparisons are expected to shed new light on the evolution of bioenergetics.

The genome sequencing programs of *C. acetobutylicum* and *C. difficile* have been largely motivated by the biotechnological and medical significance of these species. The sequence data will bring forth a more comprehensive inventory of clostridial Fe-S proteins, probably including novel ones. At this time of soaring genome sequencing programs, it is perhaps timely to reemphasize that *C. pasteurianum*, even though of limited economic significance, is worth further research because of its unusual wealth in well-characterized Fe-S proteins, and for some of its other idiosyncrasies, for instance its well established dinitrogen fixation ability. This bacterium will then certainly confirm its status as outstanding material for structural and possibly functional genomics of Fe-S proteins.

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