Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Anaerobic purification and crystallization to improve the crystal quality: ferredoxin II from *Desulfovibrio gigas*

Ying-Cheng Hsieh,[a,b] Ming-Yih Liu,[c] Jean Le Gall[c] and Chun-Jung Chen[a,b,d]*

[a]Biology Group, Research Division, National Synchrotron Radiation Research Center, Hsinchu 30077, Taiwan, [b]Department of Life Sciences and Structural Biology Program, National Tsing-Hua University, Hsinchu 30077, Taiwan, [c]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA, and [d]Department of Physics, National Tsing-Hua University, Hsinchu 30077, Taiwan

Correspondence e-mail: cjchen@nsrrc.org.tw

Received 5 August 2004
Accepted 28 December 2004

This paper is dedicated to the memory of Professor Jean Le Gall (1932–2003).

Sulfate-reducing bacteria (SRB), which are strict anaerobes, contain an electron-transfer chain from pyridine nucleotides to molecular oxygen. This unique enzymatic equipment allows the bacterium to produce ATP when exposed to air from the degradation of internal reserves of polyglucose. Ferredoxin II (Fd II) is a small electron-transfer protein isolated from the strict anaerobic sulfate-reducing bacterium *Desulfovibrio gigas*. The protein contains 58 amino acids and an iron–sulfur cluster. The cluster [3Fe–4S] spontaneously undergoes conversion to [4Fe–4S] when it is used as an electron mediator in the phosphoroclastic reaction. The iron–sulfur geometries and interconversion mechanism appear to have physiological significance between the oxidized and reduced states. Crystallization of Fd II in an anaerobic environment was achieved at a higher resolution of 1.37 Å and the differences between the anaerobic and aerobic structures will reveal the unique iron-storage function and electron-transfer mechanism of ferredoxin II from *D. gigas*.

1. Introduction

Microorganisms such as sulfate-reducing bacteria (SRB) obtain energy to grow by transferring electrons from an electron donor to an electron acceptor, such as oxygen, nitrate, iron (III), manganese (IV), sulfate, carbon dioxide *etc.* during cellular respiration (Santos *et al.*, 1993; Le Gall & Xavier, 1996). During this process (electron transport chain) the electron acceptor is reduced and the electron donor is oxidized. Despite the fact that they are still considered as strict anaerobes, when exposed to oxygen SRBs are capable of surviving (Dilling & Cypionka, 1990; Marschall *et al.*, 1993) as well as taking advantage of its presence in terms of energy conservation (Santos *et al.*, 1993; Le Gall & Xavier, 1996). In the presence of oxygen, the sulfate reducer *Desulfovibrio gigas* starts utilizing internal reserves of polyglucose, which are metabolized by the Embden–Meyerhof–Parnas pathway, thus generating NADH and ATP (Santos *et al.*, 1993; Fareleira *et al.*, 1997).

Ferredoxins (Fds), redox proteins containing Fe and S atoms, play a role in the electron-transfer processes related to the phosphoroclastic reaction and the reduction of sulfite by SRBs. These proteins of small molecular weight (∼6 kDa) have low redox potentials, characteristic electronic spectra and typical EPR signals. Seven Fds have been isolated from SRB containing four types of cluster arrangement: [3Fe–4S], [4Fe–4S], [3Fe–4S] and [4Fe–4S], and 2×[4Fe–4S] clusters. These Fds show a common structural fold because each Fe atom is tetrahedrally coordinated with bridging inorganic S atoms. Cysteinyl S atoms are generally the terminal ligands for the clusters; however, other O- and N-containing ligands such as aspartic acid may also be involved (Fukuyama *et al.*, 1980, 1989; Stout, 1988, 1989; Stout *et al.*, 1988; Kissinger *et al.*, 1989). *D. gigas* has two forms of ferredoxin, which have been termed ferredoxin I (Fd I) and ferredoxin II (Fd II). Fd I contains a single [4Fe–4S] cluster, while Fd II contains a [3Fe–4S] cluster per molecule; both proteins are composed of the same polypeptide chain (Bruschi, 1979). Fd I has a redox potential of 450 mV and Fd II exhibits an E'_o of −130 mV (Bruschi *et al.*, 1976; Cammack *et al.*, 1977).

Most Fds have a biological activity in the stimulation of hydrogen consumption with sulfite as a terminal electron acceptor or in

hydrogen production from pyruvate (Odom & Peck, 1984; Bruschi & Guerlesquin, 1988; Le Gall & Fauque, 1988; Moura *et al.*, 1984; Hatchikian & Le Gall, 1972; Le Gall & Dragoni, 1966). It has been shown that the tetraheme cytochrome c_3 is an intermediate between hydrogenase and ferredoxin (Akagi, 1967; Suh & Akagi, 1969). In *D. gigas*, Fd I and Fd II function respectively in different metabolic pathways. Fd I is required in the phosphoroclastic reaction in which hydrogen is evolved from the oxidation of pyruvate (Moura *et al.*, 1978). Fd II also stimulates this phosphoroclastic reaction after a long lag phase when it is added to Fd-depleted *D. gigas* crude extracts.

Pyruvate can induce the conversion of the [3Fe–4S] cluster of *D. gigas* Fd II into the [3Fe–4S] cluster-containing Fd I in the presence of *D. gigas* crude cell extracts (Moura *et al.*, 1984). Purified *D. gigas* Fd II can also be converted to Fd I following incubation with excess amount of Fe^{2+} in the presence of dihiothretol (Moura *et al.*, 1982; Kent *et al.*, 1982), demonstrating the polypeptide chain of *D. gigas* Fd can accommodate both [3Fe–4S] and [4Fe–4S] clusters. The [3Fe–4S]/[4Fe–4S] interconversion previously found in *D. gigas* Fds indicated that transition metals other than iron could be incorporated into the [3Fe–4S] center. *D. gigas* Fd II [Co, 3Fe–4S] was the first heterometal center ever reported (Moura *et al.*, 1986). Later, similar results were obtained from *D. africanus* Fd III (Butt, Armstrong *et al.*, 1991; Butt, Sucheta *et al.*, 1991) and *Pyrococcus furiosus* Fd (Conover, Kawal *et al.*, 1990; Conover, Park *et al.*, 1990). [Zn, 3Fe–4S] and [Ni, 3Fe–4S] centers could also be produced from *D. gigas* Fd II (Surerus *et al.*, 1987; Moreno *et al.*, 1994).

The X-ray structure analysis of aerobic *D. gigas* Fd II (Kissinger *et al.*, 1989, 1991) shows that the [3Fe–4S] cluster is bound to the polypeptide chain by three cysteinyl residues: Cys8, Cys14 and Cys50. The residue Cys11, a potential ligand for the fourth site of a [4Fe–4S] cluster, is twisted away from the cluster. A disulfide bridge between Cys18 and Cys42 is located where the second iron–sulfur cluster is found in 2×[4Fe–4S] Fds. Moreover, it was observed that this disulfide bridge can be open during the reduction of the Fd II (Macedo *et al.*, 1994). The potential role of this disulfide bridge in the physiological behavior of the Fd remains to be studied. However, the structure of [4Fe–4S] cluster-containing Fds from SRB has not been determined for the full understanding of this protein. Here, we present our experience on crystallization under an anaerobic environment and the anaerobic Fd II crystals at the high resolution of 1.37 Å and the preliminary comparison analysis of anaerobic and aerobic structures which can reveal the unique iron-storage function and electron-transfer mechanism of ferredoxin II from *D. gigas*.

2. Materials and methods

2.1. Protein purification

D. gigas was grown as described previously (Le Gall *et al.*, 1965). All purification steps of ferredoxin II were modified from Bruschi *et al.* (1976) and carried out under strict exclusion of oxygen in an anaerobic chamber (Coy Laboratory Products Inc.) filled with 95% N_2 and 5% H_2 at room temperature, unless otherwise noted. The chamber maintains a strict 0–5 parts per million (ppm) oxygen atmosphere through hydrogen gas (5% mix) reacting with a palladium catalyst to remove oxygen. An oxygen and hydrogen gas analyzer (Coy Laboratory products Inc.) was used to monitor levels of the oxygen and hydrogen in the anaerobic chambers. The detection range of the gas analyzer is 0–1999 ppm for oxygen and 0–100% for hydrogen, respectively. All buffers were prepared with oxygen-free water which was boiled for 10 min to get rid of most dissolved gas and cooled in ice. Argon was simultaneously introduced into the water through a pipe in order to avoid reintroduction of oxygen into the water. These were situated inside the anaerobic chamber for 2 d before use in order to remove the last traces of oxygen, if any. Fd II was isolated from *D. gigas* by a combination of DEAE-cellulose and gel-filtration chromatrographic procedures. The isolation of ferredoxin was largely based on its acidic characteristics. The redox potential of the purified Fd was checked. The protein purity was greater than 95% as analyzed by SDS–PAGE and the UV–visible spectrum.

Table 1
Crystal diffraction statistics of anaerobic ferredoxin II.

Values in parentheses are for the highest resolution shell (1.41–1.37 Å).

Wavelength (Å)	1.127 (BL17B2, NSRRC)
Temperature (K)	110
Resolution range (Å)	30.0–1.7
Space group	$P2_12_12$
Unique reflections	13205
Completeness (%)	94.8 (81.9)
$\langle I/\sigma(I)\rangle$	17.6 (3.0)
Average redundancy	11.2
R_{sym}† (%)	6.3 (25.2)
Mosaicity (°)	0.457
Unit-cell parameters (Å)	a = 32.69, b = 82.28, c = 22.95
No. of molecules per asymmetric unit	1

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$, where I_i is the ith measurement and $\langle I(h)\rangle$ is the weighted mean of all measurements of $I(h)$.

2.2. Crystallization

The protein sample was crystallized using the hanging-drop vapor-diffusion method performed in the anaerobic chamber at 295 K. The buffers were pre-prepared as described previously in the protein purification step. All screen and crystallization solutions were prepared outside the chamber using the special home-made device (Fig. 1) with several cycles of vacuuming degas and nitrogen filling to strictly exclude oxygen from solutions before the crystallization setup. All solutions sat inside the anaerobic chamber for at least 2 d to make sure they were oxygen-free. The initial crystallization condition containing 20%(*w*/*v*) PEG 6000 as the major precipitant was obtained using home-made screening kits. This condition was further refined to produce larger and better crystals. Hence, 1 µl drops of purified protein (~8.9 mg ml^{-1}) in 0.1 *M* MES buffer pH 6.5 were mixed with equal volumes of the reservoir solution containing 20%(*v*/*v*) PEG 6000 and 60 m*M* Zn acetate in 0.1 *M* HEPES buffer

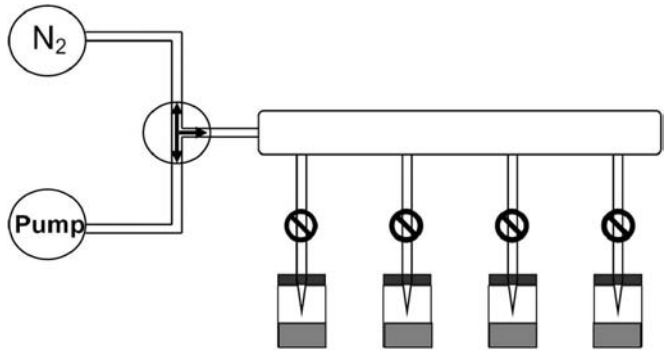

Figure 1
Oxygen-degas device. 5 ml of crystallization solution were prepared in a 15 ml bottle sealed with a rubber stopper and aluminium cap. The oxygen-degas procedure was performed as the following steps by (i) inserting the needle into the bottle through the rubber stopper, (ii) turning the valve to pump for vacuuming for 20 min, (iii) turning the valve to N_2 gas to fill with N_2 for 5 min and (iv) repeating steps (2) and (3) at least three times to ensure the O_2 is well replaced by N_2.

pH 7.0 and equilibrated against 0.5 ml reservoir solution in a 24-well ADX plate (Hampton Research, Co.). Crystals appeared after one week and slowly reached maximum size in three weeks (Fig. 2).

2.3. Crystal mounting and X-ray data collection

Crystal harvesting, mounting and freezing was performed throughout in the anaerobic chamber at 295 K. A container filled with liquid nitrogen and crystal-mounting tools were first placed inside the control box for vacuuming and nitrogen gas filling before placement into the chamber. One should be aware and extremely cautious in that the liquid nitrogen could evaporate and erupt during the vacuuming process. The best quality crystals were carefully examined under a microscope through the clear and flexible vinyl wall of the chamber and transferred into a cryoprotectant solution containing 15%(*v*/*v*) glycerol. The selected crystals were mounted on a 0.1–0.2 mm glass fiber loop (Hampton Research) and then flash-cooled in liquid nitrogen at 100 K. The liquid-nitrogen container with crystals was then taken out of the anaerobic chamber in the reverse of the above procedure. After the crystals had been taken out from the anaerobic chamber, they were constantly flushed with liquid nitrogen.

The protein crystals were initially screened and characterized using a synchrotron-radiation X-ray source at BL12B2 equipped with a Quantum 4R CCD detector (ADSC) at SPring-8 in Japan. The completion of data collection was carried out at the protein crystallographic beamline BL17B2 equipped with the Q210 CCD detector (ADSC) at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. For complete data collection, 347° rotations with 1° oscillation were measured using an X-ray wavelength of 1.127 Å with an exposure time of 60 s and a crystal-to-detector distance of 90 mm at 110 K in a nitrogen stream using the X-Stream cryo-system (Rigaku/MSC Inc.). The data were indexed, integrated, scaled and merged using the program *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Purification and crystallization experiments of the activating enzyme were carried out under strictly anaerobic conditions inside a glove box. Anaerobic Fd II crystals of rectangular shape appeared in two weeks and continued to grow slowly to a final size of 0.08 × 0.08 × 0.2 mm in three weeks in a 291 K incubator inside the chamber. The reddish crystals became colorless when exposed to oxygen for more than one week. Good quality crystals were carefully screened and selected for data collection. Attention must be paid so that the entire crystal mounting procedure may be performed as quickly as possible to avoid increasing pressure inside the chamber owing to the evaporation of liquid nitrogen. For a comparison and control experiment, the crystallization trials of the same batch of anaerobic protein solution resulted in no crystals under an aerobic environment (outside the chamber) using the same crystallization conditions for the anaerobic protein. Furthermore, the aerobically kept Fd II protein was also crystallized using similar condition with ammonium sulfate as the precipitant described previously (Kissinger *et al.*, 1991) and the crystals exhibited relatively dark-brown colors compared with anaerobic crystals.

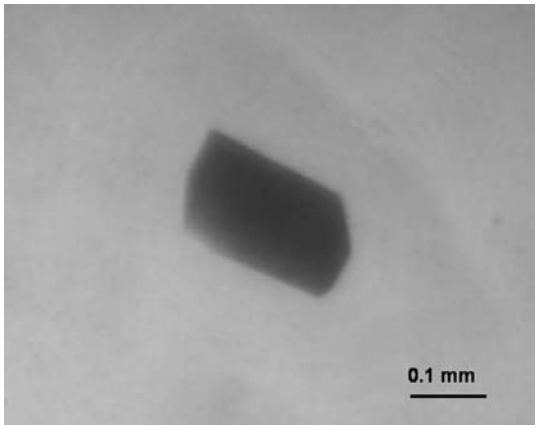

Figure 2
Single crystal of ferredoxin II grown by hanging-drop method under anaerobic conditions in the chamber.

Analysis of the diffraction pattern indicated that the crystals exhibited orthorhombic symmetry and systematic absences suggested that the space group was $P2_12_12$, with unit-cell parameters a = 32.69, b = 82.28, c = 22.95 Å. The data set was 94.8% complete with an internal agreement (R_{sym}) of 6.3% and a redundancy of 11.2 for the resolution range 30.0–1.37 Å. Assuming one molecule in the asymmetric unit, the Matthews coefficient is estimated to be 2.31 Å^3 Da^{-1}, corresponding to a solvent content of 44.74% (Matthews, 1968), which is within the normal range for protein crystals. The crystal structure of *D. gigas* Fd II with a [3Fe–4S] cluster has previously been determined with space group $C2$ at 1.7 Å resolution under aerobic conditions (Kissinger *et al.*, 1991). The special anaerobic crystallization dramatically improved the crystal quality in terms of the resolution (1.37 Å) and different space group ($P2_12_12$). Structure determination and refinement are complete and will be described in a separate structure paper.

Superimposing aerobic and anaerobic Fd II structures based on C^{α} least-squares-fit alignment shows an overall r.m.s. deviation of 1.01 Å over 58 amino acids, with the most significant main-chain structural variations occurring at residues 25–28. The tide disulfide bond (Cys18–Cys42) existing in the aerobic structure shows double conformations, interpreted as an opening up of the covalent disulfide bond. Moreover, the iron–sulfur cluster geometries in the anaerobic structure are much different from those in the aerobic structure with maximum increasing and decreasing changes of Fe—S bond distances by 0.15 and 0.14 Å ranges, respectively. Hence, the longest and shortest interatomic distances of the Fe—S bonds in the cluster are 2.18 and 2.36 Å, respectively, as the high-resolution data of 1.37 Å is feasible to refine positions of clusters atoms without restraints. The influence of these bond variations might play a role in determining the electronic configuration of the reduced cluster. Furthermore, six additional zinc metal ions are observed at the surface area ligated, with particular Glu and Asp residues implying the potential metal-binding sites for the iron and other possible heterometal-containing cluster formation and conversion in the reduced state under the metal-abundant environment. In particular, two sites with the binding residues Asp6, Asp7, Glu12 and Glu16 are relatively close to the existing cluster. Thus, the comparison between aerobic and anaerobic structures of high resolution provides valuable insights into different iron–sulfur cluster conversions and disulfide-bridge conformations as well as crystal packing, which may reveal the unique iron-storage function and electron-transfer mechanism of ferredoxin II from *D. gigas*. The detailed structure information and comparison will be described and discussed in a forthcoming separate article.

Although anaerobes outnumber aerobes in the intestinal tract by 1000:1 and even at the skin surface by 10:1 (Mortelmans, 1998), little attention has been given to their means of survival in apparently adverse conditions. The role of entire anaerobe families in human health has been largely underestimated. This lack of knowledge could cause irreparable damage in the environment and industry and

hinder human health research. This is particularly true as far as sulfate-reducing bacteria (SRBs) are concerned. Several examples have shown the physiological and structural differences of proteins from SRBs present in both anaerobic and aerobic environment, such as the zinc movement related to the enzymatic activity of rubrerythrin from *D. vulgaris* under conditions of different oxygen levels (Li *et al.*, 2003) and the intact green protein of a single peptide chain from *Bacillus halodenitrificans* simultaneously dissociated into two major components manganese superoxide dismutase (Liao *et al.*, 2002) and nucleoside diphosphate kinase (Chen *et al.*, 2003) when exposed to oxygen. Thus, it then becomes essential to understand how these bacteria can proliferate or survive in adverse conditions once being exposed to molecular oxygen, and the biochemical knowledge of these pathways can provide new targets to control their proliferation. As the importance of anaerobic strains increases, the anaerobic protein crystallography provides the possibility of working with oxygen-sensitive compounds and proteins for structure–function studies to fully understand their unique metabolism pathways and life cycles.

We are grateful to our colleagues and staff for valuable technical assistance and discussions during synchrotron-radiation X-ray facility data collection at BL17B2 of NSRRC in Taiwan and BL12B2 of SPring-8 in Japan. This study was supported in part by the National Synchrotron Radiation Research Center grants 924RSB02, 934RSB02 and National Science Council grant NSC-92-2321-B-213-001, Taiwan to C-JC.

References

Akagi, J. M. (1967). *J. Biol. Chem.* **242**, 2478–2483.
Bruschi, M. (1979). *Biochem. Biophys. Res. Commun*. **91**, 623–628.
Bruschi, M. & Guerlesquin, F. (1988). *FEMS Microbiol. Lett.* **54**, 155–176.
Bruschi, M., Hatchikian, E. C., Le Gall, J., Moura, J. J. G. & Xavier, A. V. (1976). *Biochim. Biophys. Acta*, **499**, 275–284.
Butt, J. N., Armstrong, F. A., Breton, J., George, S. J., Thomson, A. J. & Hatchikian, E. C. (1991). *J. Am. Chem. Soc.* **113**, 6663–6670.
Butt, J. N., Sucheta, A., Armstrong, F. A., Breton, J., Thomson, A. J. & Hatchikian, E. C. (1991). *J. Am. Chem. Soc.* **113**, 8948–8950.
Cammack, R., Rao, K. K., Hall, D. O., Moura, J. J. G., Xavier, A. V., Bruschi, M., Le Gall, J., Deville, A. & Gayda, J.-P. (1977). *Biochim. Biophys. Acta*, **490**, 311–321.
Chen, C.-J., Liu, M.-Y., Chang, T., Chang, W.-C., Wang, B.-C. & Le Gall, J. (2003). *J. Struct. Biol.* **142**, 247–255.
Conover, R. C., Kawal, A. T., Fu, W., Park, J.-B., Aono, S., Adams, M. W. W. & Johnson, M. K. (1990). *J. Biol. Chem.* **265**, 8533–8541.
Conover, R. C., Park, J.-B., Adams, M. W. W. & Johnson, M. K. (1990). *J. Am. Chem. Soc.* **112**, 4562–4564.
Dilling, W. & Cypionka, H. (1990). *FEMS Microbiol. Lett.* **71**, 123–128.
Fareleira, P., Le Gall, J., Xavier, A. V. & Santos, H. (1997). *J. Bacteriol.* **179**, 3972–3980.
Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M, Wada, K. & Matsubara, H. (1980). *Nature (London)*, **286**, 522–524.
Fukuyama, K., Tsukihara, T. & Katsube, Y. (1989). *J. Mol. Biol.* **210**, 383–398.
Hatchikian, E. C. & Le Gall, J. (1972). *Biochim. Biophys. Acta*, **267**, 479–484.
Kent, T. A., Moura, I., Moura, J. J. G., Lipscomb, J. D., Huynh, B. H., Le Gall, J., Xavier, A. V. & Münck, E. (1982). *FEBS Lett.* **138**, 55–58.
Kissinger, C. R., Adman, E. T., Sieker, L. C., Jensen, L. H. & Le Gall, J. (1989). *FEBS Lett.* **244**, 447–450.
Kissinger, C. R., Sieker, L. C., Adman, E. T. & Jensen, L. H. (1991). *J. Mol. Biol.* **219**, 693–715.
Le Gall, J. & Dragoni, N. (1966). *Biochem. Biophys. Res. Commun*. **23**, 145–149.
Le Gall, J. & Fauque, G. (1988). *Biology of Anaerobic Microorganisms*, pp. 587–639. New York: John Wiley & Sons.
Le Gall, J., Mazza, G. & Dragoni, N. (1965). *Biochim. Biophys. Acta*, **99**, 385–387.
Le Gall, J. & Xavier, A.V. (1996). *Anaerobe*, **2**, 1–9.
Li, M., Liu, M.-Y., Le Gall, J., Gui, L.-L., Liao, J., Jiang, T., Zhang, J.-P., Liang, D.-C. & Chang, W.-R. (2003). *J. Biol. Inorg. Chem.* **8**, 149–155.
Liao, J., Liu, M. Y., Chang, T., Li, M., Le Gall, J., Gui, L.-L., Zhang, J.-P., Jiang, T., Liang, D.-C. & Chang, W.-R. (2002). *J. Struct. Biol.* **139**, 171–180.
Macedo, A. L., Moura, I., Surerus, K. K., Papaefthymiou, V., Liu, M.-Y., Le Gall, J., Munck, E. & Moura, J. J. G. (1994). *J. Biol. Chem.* **268**, 8052–8058.
Marschall, C., Frenzel, P. & Cypionka, H. (1993). *Arch. Microbiol.* **159**, 168–173.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Moura, I., Moura, J. J. G., Munck, E., Papaefthymiou, V. & Le Gall, J. (1986). *J. Am. Chem. Soc.* **108**, 349–351.
Moura, J. J. G., Cavier, A. V., Hatchikian, E. C. & Le Gall, J. (1978). *FEBS Lett.* **89**, 177–179.
Moura, J. J. G., Le Gall, J. & Xavier, A. V. (1984). *Eur. J. Biochem.* **141**, 319–322.
Moura, J. J. G., Moura, I., Kent, T. A., Lipscomb, J. D., Huynh, B. H., Le Gall, J., Xavier, A. V. & Münck, E. (1982). *J. Biol. Chem.* **257**, 6259–6267.
Moreno, C., Macedo, A. L., Moura, I., Le Gall, J. & Moura, J. J. G. (1994). *J. Inorg. Biochem.* **53**, 219–234.
Mortelmans, K. (1998). *SIM News*, **48**, 261–265.
Odom, J. M. & Peck, H. D. Jr (1984). *Annu. Rev. Microbiol.* **38**, 551–592.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Santos, H., Fareleira, P., Xavier, A. V., Chen, L., Liu, M.-Y. & Le Gall, J. (1993). *Biochem. Biophys. Res. Commun*. **195**, 551–557.
Stout, C. D. (1988). *J. Biol. Chem.* **263**, 9256–9260.
Stout, C. D. (1989). *J. Mol. Biol.* **205**, 545–555.
Stout, G. H., Turley, S., Sieker, L. C. & Jenson, L. H. (1988). *Proc. Natl Acad. Sci. USA*, **85**, 1020–1027.
Suh, B. & Akagi, J. M. (1969). *J. Bacteriol.* **99**, 210–215.
Surerus, K. K., Münck, E., Moura, I., Moura, J. J. G. & Le Gall, J. (1987). *J. Am. Chem. Soc.* **109**, 3805–3807.