Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Structures of complexes of octahaem cytochrome *c* nitrite reductase from *Thioalkalivibrio nitratireducens* with sulfite and cyanide

Anton A. Trofimov,[a]* Konstantin M. Polyakov,[a,b] Konstantin M. Boyko,[b] Tamara V. Tikhonova,[b] Tatyana N. Safonova,[b] Alexey V. Tikhonov,[b] Alexandre N. Popov[c] and Vladimir O. Popov[b]

[a]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, Moscow 119991, Russia, [b]Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr. 33, Moscow 119071, Russia, and [c]European Molecular Biology Laboratory (EMBL), c/o DESY, Notkestrasse 85, 22603 Hamburg, Germany

Correspondence e-mail: kostya@eimb.ru

Received 10 June 2010
Accepted 6 August 2010

PDB References:
octahaem cytochrome *c* nitrite reductase, complex with sulfite, 3fo3; complex with cyanide, 3mmo.

The structures of complexes of octahaem cytochrome *c* nitrite reductase from the bacterium *Thioalkalivibrio nitratireducens* (TvNiR) with the substrate sulfite (1.4 Å resolution; $R_{\rm cryst}$ = 0.126) and the inhibitor cyanide (1.55 Å resolution; $R_{\rm cryst}$ = 0.148) have been established. The complex with sulfite was prepared by the reduction of the protein crystal with sodium dithionite. The sulfite ion is bound to the iron ion of the catalytic haem through the S atom. The Fe—S distance is 2.24 Å. The structure of the cyanide complex with full occupancy of the ligand site was established for the first time for cytochrome *c* nitrite reductases. The cyanide ion is bound to the catalytic haem iron through the C atom. The Fe—C distance is 1.91 Å and the Fe—C—N angle is 171°. The sulfite reductase activity of TvNiR was measured at different pH values. The activity is 0.02 µmol of HS^- per minute per milligram at pH 7.0; it decreases with increasing pH and is absent at pH 9.0.

1. Introduction

Octahaem cytochrome *c* nitrite reductase isolated from the haloalkaliphilic γ-proteobacterium *Thioalkalivibrio nitratireducens* (TvNiR) catalyzes the reduction of nitrite and hydroxylamine to ammonia (Tikhonova *et al.*, 2006). The nitrite reductase activity of TvNiR is higher than those of the NrfA pentahaem cytochrome *c* nitrite reductases (Einsle *et al.*, 1999), which are structurally similar to TvNiR (Polyakov *et al.*, 2009). The structures of free TvNiR and of the complexes of the enzyme with the substrate nitrite and the inhibitors azide (Polyakov *et al.*, 2009) and phosphate (Trofimov *et al.*, 2010) have previously been established.

The TvNiR monomer (molecular weight 64 kDa) consists of two domains. The N-terminal domain binds the first three haem groups and has a unique tertiary structure. The second catalytic domain binds five haem groups and its tertiary structure is similar to that of the NrfA monomer. The haem groups of the catalytic domain of TvNiR superimpose well with the haem groups of NrfA, including the active-site haem. The active sites of TvNiR and NrfA are formed by the lysine-coordinated haem and Arg, Tyr and His residues. In the TvNiR and NrfA structures a calcium ion is located near the active site. The calcium ion is in a conserved environment and plays a structuring role. An important difference between TvNiR and NrfA is that there is a covalent bond in TvNiR between the CE2 atom of the catalytic residue Tyr303 and the S atom of the adjacent residue Cys305, which replaces the phenylalanine found in NrfA. It has been supposed that the

appearance of this bond in the active site of the cytochrome *c* nitrite reductase results in an increase in the nitrite reductase activity of the enzyme (Polyakov *et al.*, 2009). The quaternary structures of TvNiR and NrfA are also different. The TvNiR monomers form hexameric molecules of 32 point symmetry both in solution and in crystals, whereas the NrfA monomers form dimeric molecules.

Despite its high nitrite reductase activity, TvNiR is probably not responsible for nitrite reduction in cells, as anaerobic cell growth in the presence of nitrate leads to the accumulation of nitrite (Sorokin *et al.*, 2003). Based on the facts that TvNiR is present in cells at an amount corresponding to 10% of the total amount of soluble protein, that sulfur compounds play an important role in the life cycle of *T. nitratireducens* (unpublished data) and that TvNiR can reduce sulfite to sulfide, which has been qualitatively shown previously (Tikhonova *et al.*, 2006), we speculated that TvNiR could be a sulfite reductase in the bacterium *T. nitratireducens*.

The sulfite reductase activities of NrfAs (Table 2) are higher than those determined for some sirohaem-containing sulfite reductases. For NrfA the structure of the complex with sulfite has been determined, the catalytic tyrosine residue has been shown to play an important role in the manifestation of the nitrite reductase activity and the mutation of this residue to phenylalanine has been shown to have no effect on the sulfite reductase activity (Lukat *et al.*, 2008).

In the present study, the sulfite reductase activity of TvNiR was quantitatively characterized and the structures of the complexes of TvNiR with sulfite (TvNiR–SO_3) and cyanide (TvNiR–CN), which is the most efficient inhibitor of the enzyme (Tikhonova *et al.*, 2006), were established. It should be noted that structural evidence is lacking for the binding of cyanide in the active site of NrfA.

Table 1
Data-collection and refinement statistics for the TvNiR complexes.

Values in parentheses are for the highest resolution shell.

	TvNiR–SO_3	TvNiR–CN
PDB code	3fo3	3mmo
Space group	$P2_13$	$P2_13$
Unit-cell parameter (Å)	193.79	194.56
Molecules per asymmetric unit	2	2
X-ray source	DESY BW7B	DESY X11
Wavelength (Å)	0.843	0.810
Resolution (Å)	1.40 (1.42–1.40)	1.55 (1.57–1.55)
No. of unique reflections	468027	350257
Completeness (%)	99.7 (100)	99.9 (100)
Multiplicity	5.6 (5.6)	4.9 (4.8)
Mean $I/\sigma(I)$	33.1 (2.7)	40.1 (3.9)
$R_{\rm merge}$†	0.055 (0.547)	0.047 (0.421)
B factor from Wilson plot (Å^2)	21.8	25.1
$R_{\rm cryst}$	0.126	0.148
$R_{\rm free}$	0.140	0.162
Root-mean-square deviations		
Bond lengths (Å)	0.018	0.020
Bond angles (°)	1.558	1.609
No. of protein residues/atoms	1038/8344	1036/8318
No. of ligand atoms		
Haem groups	704	688
Other ligands	238	138
No. of water molecules	1385	1218
Average B factor (Å^2)		
All atoms	18.5	18.9
Water molecules	27.9	27.8
DPI‡	0.032	0.044
Ramachandran plot statistics, residues in (%)		
Most favoured regions	88.7	88.9
Additionally allowed regions	10.7	10.7
Generously allowed regions	0.6	0.4
Disallowed regions	0	0

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ Cruickshank's DPI calculated by *REFMAC*5 (Murshudov *et al.*, 1997).

2. Materials and methods

2.1. Enzyme-activity measurements

The catalytic reduction of sulfite was carried out in an anaerobic box at a residual oxygen pressure of at most 2 p.p.m. at 293 K in 0.05 *M* potassium phosphate buffer. Methyl viologen (MV) pre-reduced with europium(II) chloride was used as the electron donor. The TvNiR concentration was 0.06 mg ml^{-1}. The reaction was initiated by the addition of sodium sulfite. The initial concentration of sodium sulfite was 4.5 m*M*.

The sulfite reductase activity of TvNiR was determined in two ways. Firstly, the activity was determined from the rate of oxidation of MV measured spectrophotometrically from the decrease in the absorbance of the reaction mixture at λ = 601 nm. Secondly, the activity was measured from the rate of formation of the reaction product (sulfide ion). The reaction was terminated by the addition of concentrated sodium hydroxide solution. The concentration of sulfide ions was measured in air from the formation of methylene blue according to a known procedure (Cline, 1969) modified as follows: a 60 µl sample containing sulfide was added to 140 µl 0.05 *M* zinc acetate solution, the mixture was shaken until the blue colour of MV disappeared and the diamine reagent (30 µl; Cline, 1969) was added (without stirring). After 15 min, 500 µl water was added and after exactly 45 min the absorbance of the solution was measured at λ = 665 nm. The concentration of sulfide was calculated from the calibration straight line.

2.2. Complex preparation

The protein was isolated and purified as described previously (Tikhonova *et al.*, 2006). The free enzyme was crystallized by the hanging-drop vapour-diffusion method at room temperature. The drops were prepared by mixing equal volumes (5 µl) of the protein solution and reservoir solution. The protein solution was composed of 14.5 mg ml^{-1} TvNiR, 0.02 *M* sodium tetraborate and 0.05 *M* Tris–HCl pH 8.7. The reservoir solution contained 0.2 *M* trisodium citrate dihydrate, 0.1 *M* Tris–HCl pH 8.5 and 30%(*v*/*v*) PEG 400 (Crystal Screen Cryo condition No. 13; Hampton Research).

The complex of TvNiR with cyanide was prepared by soaking a protein crystal in a solution containing 0.1 *M* potassium cyanide, 0.2 *M* trisodium citrate dihydrate, 0.1 *M* Tris–HCl pH 8.5 and 30%(*v*/*v*) PEG 400 for 1 h.

Table 2
Sulfite reductase activities of TvNiR, SiR and NrfA.

Enzyme	Activity† (μmol H_2 min^{-1} mg^{-1})		
	pH 7.0	pH 7.8	pH 9.0
TvNiR	0.065	0.013	None
SiR from *D. desulfuricans* (Steuber *et al.*, 1995)	0.041	—	—
NrfA from *W. succinogenes* (Lukat *et al.*, 2008)	0.4	—	—
NrfA from *Sulfurospirillum deleyianum* (Stach *et al.*, 2000)	1.1	—	—
NrfA from *D. desulfuricans* (Pereira *et al.*, 1996)	2.06	—	—

† For the convenience of comparison with the published data, the activities of TvNiR are given in terms of H_2 units.

The complex of TvNiR with sulfite was prepared by soaking a protein crystal in a solution containing 0.1 *M* sodium dithionite, 0.2 *M* trisodium citrate dihydrate, 0.1 *M* Tris–HCl pH 8.5 and 30%(*v*/*v*) PEG 400 for 10 min.

2.3. Data collection and refinement

The X-ray diffraction data sets for the complexes of TvNiR with cyanide and sulfite were collected at the EMBL Hamburg Outstation using CCD detectors at 100 K. The data were processed using the *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997).

The structure of free TvNiR (PDB code 2ot4; Polyakov *et al.*, 2009) was used as the starting model for structure refinement of the complexes. Refinement was carried out using the *REFMAC*5 software (Murshudov *et al.*, 1997) with simultaneous minimization of the X-ray and geometric terms. The structure of the TvNiR–SO_3 complex was refined with anisotropic displacement parameters for all non-H atoms. In the refinement of the TvNiR–CN complex only the haem iron ions were refined anisotropically using the default sphericity restraint on the atomic anisotropic tensors. The graphics program *Coot* (Emsley & Cowtan, 2004) was used for manual model building based on electron-density maps. The data-collection and refinement statistics are given in Table 1. The average *B* factors were calculated with *BAVERAGE* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

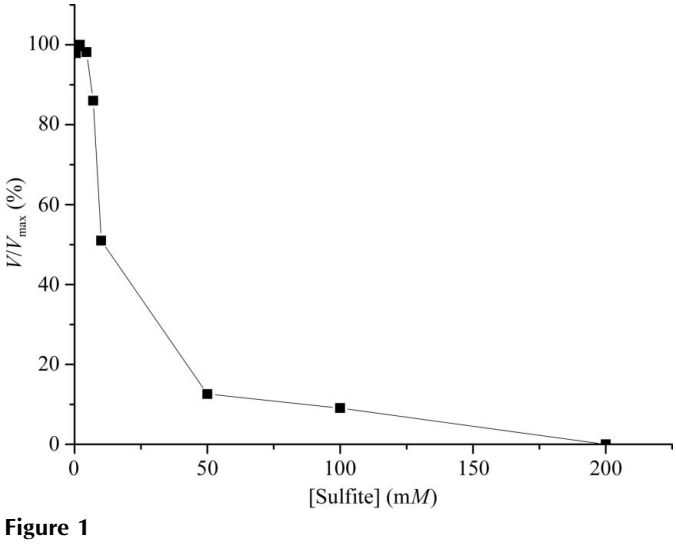

Figure 1
Inhibition of the sulfite reductase activity of TvNiR at high sulfite concentrations.

Table 3
Interatomic distances and *B* factors (average values for monomers *A* and *B*) for the atoms in the active sites of the TvNiR complexes.

B factors (Å^2) are given in parentheses. The average difference between the *B* factors of these atoms in monomers *A* and *B* was 0.7 Å^2 for the TvNiR–SO_3 complex and 1.3 Å^2 for the TvNiR–CN complex.

Atom *i*	Atom *j*	Distance (Å)	
		Molecule *A*	Molecule *B*
TvNiR–SO_3			
Haem4 Fe (13)	SO_3 S (14)	2.24	2.23
Haem4 Fe (13)	Lys188 NZ (15)	1.94	1.93
Tyr303 OH (16)	SO_3 O2 (15)	2.57	2.53
His361 NE2 (13)	SO_3 O2 (15)	2.74	2.75
Arg131 NH2 (14)	SO_3 O3 (18)	2.96	2.92
W1 (18)	SO_3 O1 (18)	2.48	2.48
W2 (14)†	SO_3 O3 (18)	2.68	2.68
W3 (18)†	SO_3 O3 (18)	2.98	2.91
TvNiR–CN			
Haem4 Fe (13)	CN C (13)	1.91	1.91
Haem4 Fe (13)	Lys188 NZ (15)	1.97	1.96
Tyr303 OH (16)	CN N (17)	2.77	2.76
His361 NE2 (14)	CN N (17)	3.12	3.15
W1 (15)	CN N (17)	4.15	4.17
W2 (22)	CN N (17)	3.62	3.57
W3 (19)	CN N (17)	4.00	3.96
W4 (15)	CN N (17)	2.81	2.81

† The occupancies of water molecules W2 and W3 were 0.3 and 0.7, respectively.

3. Results and discussion

The sulfite reductase activity of TvNiR at pH 7.0 and 293 K measured according to the first and second procedures was 0.13 ± 0.02 μmol MV per minute per milligram of TvNiR and 0.02 ± 0.005 μmol HS^- per minute per milligram of TvNiR, respectively. These data confirm the six-electron reduction of sulfite to sulfide. The sulfite reductase activity of TvNiR has a maximum value at neutral pH. The activity measured at pH 7.8 is 20% of the activity measured at pH 7.0. Sulfite reductase activity was not detected at pH 9.0. Starting with a sulfite concentration of 6–7 m*M*, the rate of the catalytic reaction of TvNiR was found to decrease as the concentration of the substrate increased (Fig. 1). At a sulfite concentration of 0.2 *M* the catalytic reaction was completely inhibited.

The sulfite reductase activity of TvNiR is lower than those of NrfAs (Table 2). The activity of TvNiR is comparable to that of the sirohaem-containing sulfite reductase from *Desulfovibrio desulfuricans* (SiR; Steuber *et al.*, 1995).

Overall, the structures of the TvNiR–SO_3 and TvNiR–CN complexes are similar to that of free TvNiR (Polyakov *et al.*, 2009). The crystallographically independent monomers in the asymmetric unit cell (monomers *A* and *B*) of the structure of TvNiR superimpose with an r.m.s. deviation of about 0.2 Å on all C^α atoms, as do any of the monomers from the various structures. The side chains of the catalytic residues Arg131,

Tyr303 and His361 and the catalytic haems of the complexes and the free form of TvNiR superimpose with an r.m.s. deviation of about 0.1 Å on all atoms. In contrast to the structure of the free form of the enzyme, the side chain of the Gln360 residue in the structures of the TvNiR–SO_3 and TvNiR–CN complexes, like the corresponding side chain in the structures of the previously solved complexes, has one conformation, in which the OE1 atom of the residue coordinates the calcium ion near the active site. In the structure of the free form of the enzyme one of the positions of the glutamine residue coincides with that mentioned above, whereas the side chain of the residue in the other conformation is not involved in the coordination of calcium and points towards the catalytic haem.

The active site of the TvNiR–SO_3 complex contains a sulfite ion (Fig. 2) which is bound to the catalytic haem iron through the S atom and forms five hydrogen bonds. The contacts of the sulfite ion with the adjacent atoms are listed in Table 3. The O1 atom of the sulfite interacts with water molecule W1, which is bound to the carboxyl groups of the catalytic haem. The O2 atom interacts with the NE2 atom of His361 and the hydroxyl group of Tyr303. The protonated state of the imidazole group of the histidine can be attributed to proton generation during the oxidation of dithionite. The O3 atom of the sulfite forms hydrogen bonds to the NH2 atom of Arg131 and one water molecule (either W2 or W3). These water sites are partially occupied and only one of the water molecules can be present in the structure. Water molecule W2 forms a hydrogen bond to the NE atom of Arg131 and water molecule W3 forms a hydrogen bond to the hydroxyl group of Tyr303. An analogous binding pattern was observed for the sulfite in the structure of the complex of NrfA from *Wolinella succinogenes* (PDB code 3bnf; Lukat *et al.*, 2008), with the only difference being that water molecule W3 was absent at the entrance to the active site and the W2 site was fully occupied.

The active site of the TvNiR–CN complex contains the cyanide ion bound to the haem iron through the C atom (Fig. 3, Table 3). The cyanide ion is almost perpendicular to the pyrrole–pyrroline plane of the haem. The Fe—C—N angle is 171°, which is similar to this angle in the complexes of haem-containing proteins with cyanide; for example, sulfite reductase (Fe—C—N angle of 171°; PDB code 4gep; Crane *et al.*, 1997) and peroxidase (Fe—C—N angle of 170°; PDB code 2e39; Fukuyama & Okada, 2007). The N atom of the cyanide forms hydrogen bonds to the hydroxyl group of Tyr303 and water molecule W4, which is absent from the structure of the TvNiR–SO_3 complex but is present in the structure of the free enzyme. The distance between the N atom of the cyanide and the NE2 atom of His361 is 3.1 Å. These atoms are not linked by a hydrogen bond because of the deprotonated state of the imidazole moiety of the histidine residue at pH 8.7. Water molecules W2 and W3 have full occupancy in the structure of the TvNiR–CN complex.

As expected, the catalytic reaction of TvNiR is inhibited by cyanide because the latter is directly bound to the catalytic haem iron. In the structure of the TvNiR complex with azide, the inhibitor azide is also bound to the catalytic haem iron (Polyakov *et al.*, 2009). For NrfA, it was shown that azide ion inhibits catalysis because of its binding at the entrance to the active site (Einsle *et al.*, 2000).

According to the hard and soft acids and bases principle, sulfite and cyanide ions should interact better with the haem iron ion in the reduced state compared with the oxidized state. This accounts for the rapid replacement of phosphate ions by cyanide in the active sites of sirohaem-containing sulfite reductase upon its reduction, whereas the phosphates are only replaced after many days when the protein is in the oxidized state (Crane *et al.*, 1997). Previously, we have only succeeded in replacing phosphate with cyanide in half of the active sites of TvNiR by soaking a crystal of the phosphate complex of the enzyme in 0.1 *M* potassium cyanide solution for 7 h (Trofimov *et al.*, 2010). In the present study, we obtained a complex of TvNiR with cyanide with full occupancy of the ligand owing to the use of the free form of the enzyme in the soaking.

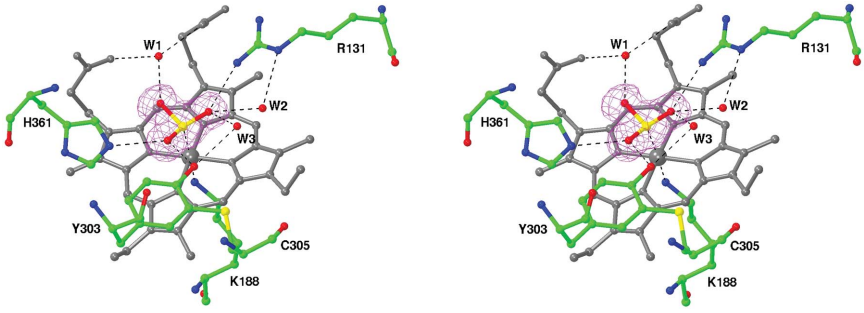

Figure 2
Binding of the sulfite ion in the active site of TvNiR. The OMIT electron-density map for the sulfite ion is shown (6σ). The electron-density map corresponds exactly to the $2F_o - F_c$ map in this region contoured at 1σ. Hydrogen bonds and coordination bonds are indicated by dashed lines.

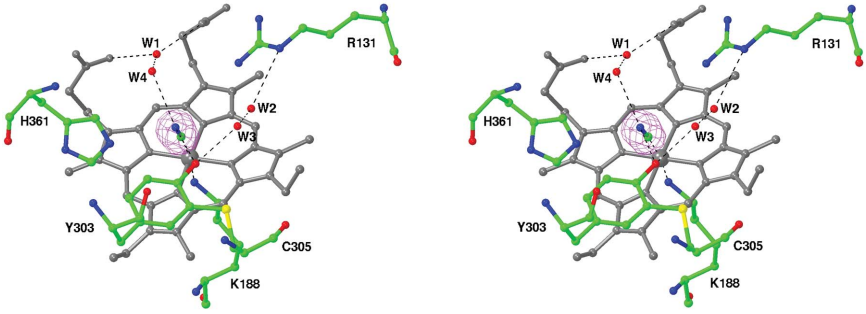

Figure 3
Binding of the cyanide ion in the active site of TvNiR. The OMIT electron-density map for the cyanide ion is shown (6σ). The electron-density map exactly corresponds to the $2F_o - F_c$ map in this region contoured at 1σ. Hydrogen bonds and coordination bonds are indicated by dashed lines.

The TvNiR–SO_3 complex was obtained rapidly and with full occupancy of the ligand because of the reduction of the protein. In this case, the sulfite ion is the oxidation product of dithionite, which is known to reduce TvNiR in solution (Tikhonova *et al.*, 2006). The reduction of the catalytic haem in TvNiR on treatment with dithionite can be demonstrated by the complete replacement of the phosphate ions by sulfite in the active sites of the enzyme after soaking a crystal of the TvNiR complex with phosphate in 0.1 *M* sodium dithionite solution for 10 min (structure at 1.67 Å resolution; $R_{\rm cryst}$ = 0.172). The shorter Fe—S distance in the TvNiR–SO_3 complex (2.24 Å) compared with that in the sulfite complex of NrfA (2.28 Å; Lukat *et al.*, 2008) may be associated with the reduced state of the catalytic haem in the former case, but the difference in the Fe—S distance in these structures is within the experimental error in the determination of the atomic coordinates.

The fact that TvNiR does not exhibit sulfite reductase activity at pH 9.0 is attributed to both a low concentration of the protons necessary for the transformation of sulfite into sulfide and weaker binding of the sulfite ions in the active site of the enzyme owing to the deprotonation of His361 and Tyr303 at pH 9.0. At alkaline pH, water molecule W1 bound to the deprotonated carboxyl groups of the haem can also hinder the interaction of the ligands with the catalytic haem iron. We failed to prepare a complex of TvNiR with a strictly determined position of the sulfite ion by soaking a protein crystal in sodium sulfite solution at pH 8.7 (PDB code 3f29; A. A. Trofimov, K. M. Polyakov, T. V. Tikhonova & V. O. Popov, unpublished work). It is probable that the decrease in the sulfite reductase activity of TvNiR at high concentrations of the substrate is associated with competition of the negatively charged product (HS^-) and substrate for the positively charged substrate channel, although sulfite anions were not found in the substrate channel in the structure of the TvNiR–SO_3 complex.

The low sulfite reductase activity of TvNiR and the absence of activity at alkaline pH (the physiological pH for the enzyme) suggest that reduction of sulfite to sulfide is not the role of TvNiR in cells.

This research was supported by grants N 10-04-01695 and N 08-04-00830-a from the Russian Foundation for Basic Research.

References

Cline, J. D. (1969). *Limnol. Oceanogr.* **14**, 454–458.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Crane, B. R., Siegel, L. M. & Getzoff, E. D. (1997). *Biochemistry*, **36**, 12120–12137.
Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G. P., Bartunik, H. D., Huber, R. & Kroneck, P. M. H. (1999). *Nature (London)*, **400**, 476–480.
Einsle, O., Stach, P., Messerschmidt, A., Simon, J., Kroger, A., Huber, R. & Kroneck, P. M. H. (2000). *J. Biol. Chem.* **275**, 39608–39616.
Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* D**60**, 2126–2132.
Fukuyama, K. & Okada, T. (2007). *Acta Cryst.* D**63**, 472–477.
Lukat, P., Rudolf, M., Stach, P., Messerschmidt, A., Kroneck, P. M. H., Simon, J. & Einsle, O. (2008). *Biochemistry*, **47**, 2080–2086.
Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pereira, I. C., Abreu, I. A., Xavier, A. V., LeGall, J. & Teixeira, M. (1996). *Biochem. Biophys. Res. Commun.* **224**, 611–618.
Polyakov, K. M., Boyko, K. M., Tikhonova, T. V., Slutsky, A., Antipov, A. N., Zvyagilskaya, R. A., Popov, A. N., Bourenkov, G. P., Lamzin, V. S. & Popov, V. O. (2009). *J. Mol. Biol.* **389**, 846–862.
Sorokin, D. Y., Touruva, T. P., Sjollema, K. A. & Kuenen, J. G. (2003). *Int. J. Syst. Evol. Microbiol.* **53**, 1779–1783.
Stach, P., Einsle, O., Schumacher, W., Kurun, E. & Kroneck, P. M. H. (2000). *J. Inorg. Biochem.* **79**, 381–385.
Steuber, J., Arendsen, A. F., Hagen, W. R. & Kroneck, P. M. H. (1995). *Eur. J. Biochem.* **233**, 873–879.
Tikhonova, T. V., Slutsky, A., Antipov, A. N., Boyko, K. M., Polyakov, K. M., Sorokin, D. Y., Zvyagilskaya, R. A. & Popov, V. O. (2006). *Biochim. Biophys. Acta*, **1764**, 715–723.
Trofimov, A. A., Polyakov, K. M., Boyko, K. M., Filimonenkov, A. A., Dorovatovskii, P. V., Tikhonova, T. V., Popov, V. O. & Kovalchuk, M. V. (2010). *Crystallogr. Rep.* **55**, 58–64.