Structure at 1.3 Å Resolution of Rhodothermus marinus caa₃ Cytochrome c Domain

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The cytochrome c domain of subunit II from the Rhodothermus marinus caa₃ HiPIP:oxygen oxidoreductase, a member of the superfamily of heme-copper-containing terminal oxidases, was produced in Escherichia coli and characterised. The recombinant protein, which shows the same optical absorption and redox properties as the corresponding domain in the holo enzyme, was crystallized and its structure was determined to a resolution of 1.3 Å by the multiwavelength anomalous dispersion (MAD) technique using the anomalous dispersion of the heme iron atom. The model was refined to final R cryst and R free values of 13.9% and 16.7%, respectively. The structure reveals the insertion of two short antiparallel b-strands forming a small b-sheet, an interesting variation of the classical all a-helical cytochrome c fold. This modification appears to be common to all known caa₃-type terminal oxidases, as judged by comparative modelling and by analyses of the available amino acid sequences for these enzymes. This is the first high-resolution crystal structure reported for a cytochrome c domain of a caa₃-type terminal oxidase. The R. marinus caa₃ uses HiPIP as the redox partner. The calculation of the electrostatic potential at the molecular surface of this extra C-terminal domain provides insights into the binding to its redox partner on one side and its interaction with the remaining subunit II on the other side.

Keywords: cytochrome c; R. marinus; caa₃ oxygen oxidoreductases; multiwavelength anomalous dispersion; crystal structure

Introduction

The terminal enzymatic complexes of aerobic respiratory chains use the electrons of substrates like cytochrome c, ubiquinol or even high-potential iron sulphur proteins (HiPIPs) to reduce molecular oxygen to water. Most of these terminal oxidases belong to the superfamily of heme-copper-containing terminal oxidases, which carry a low spin heme and a binuclear heme-copper centre in subunit I.1-3 The reduction of oxygen to water takes place at this binuclear centre. Concomitantly, an electrochemical potential is established, because the electrons and protons necessary for the reaction originate from opposite sides of the cytoplasmic (in the case of prokaryotes) or mitochondrial membranes. Moreover, the heme-copper-containing terminal oxidases translocate (“pump”) additional protons across the membrane, thereby further enhancing the electrochemical proton potential. This potential is then used for the synthesis of ATP by ATP synthase, the uptake of ions and nutrients as well as for driving the rotation of the flagellar motor.

In order to allow the transfer of protons to the catalytic centre and to the other side of the membrane, heme-copper-containing terminal oxidases possess intra-protein proton transfer pathways. Such proton transfer pathways have been identified in subunit I by X-ray crystallography,4,5 on the basis of the results of site-directed mutagenesis experiments.6,7 They are called D-pathway and K-pathway, because an aspartate residue or a lysine residue play an important role in these
pathways. Sequence signatures of the electron transfer pathways have been used to classify the heme-copper-containing terminal oxidases into three families, called A, B and C.8,9

Besides the catalytic subunit I, the heme-copper-containing terminal oxidases possess at least a second subunit as part of their minimal functional units. Those terminal oxidases of families A and B whose electron donors are hydrophilic proteins such as cytochrome c, HiPIPs or copper proteins, have a binuclear copper centre, CuA, in subunit II, while quinol oxidising enzymes do not. Some terminal oxidases besides CuA also contain a C-type heme in their subunit II. The caa3 HiPIP:oxygen oxidoreductase from Rhodothermus marinus is one example of a heme-copper-containing terminal oxidase, which belongs to the A2 family, as indicated by the presence of a YS sequence motif in its subunit I D-pathway, containing in its subunit II a CuA centre and a C-type heme.10,11 In spite of this modification in the D-pathway, namely the absence of a glutamate residue (E278 in the case of Paracoccus denitrificans) present in A-type terminal oxidases, these enzymes are canonical proton pumps.12,13 Furthermore, A2 enzymes are not specifically related to thermophily or to particular phylogenetic groups.8,14 A homology model was derived for the R. marinus caa3 cytochrome c domain of subunit II, and interestingly this domain could only be modelled using as templates molecules from two cytochrome c families: the N-terminal region from the cytochrome c and the C-terminal one from the cytochrome c5 families,15 indicating the addition of a new structural element to the classical cytochrome c fold in this protein.

In order to elucidate this interesting observation and to get better insights into the function, the gene fragment coding for the cytochrome c domain of R. marinus caa3 HiPIP:oxygen oxidoreductase was cloned into an expression vector and overexpressed. The protein fragment obtained was purified, characterized and crystallised. The crystals diffracted to the very high resolution of 1.3 Å, and the structure was determined by the multiwavelength anomalous dispersion (MAD) technique. The biochemical characterization and the structural features for the docking of the cytochrome c domain with the redox partner and the remaining subunit II are presented here.

Results

Biochemical characterization

The cytochrome c domain of subunit II from the R. marinus caa3 HiPIP:oxygen oxidoreductase was successfully expressed in Escherichia coli, using the cytochrome c maturation genes of the pEC86 vector. From 10 l of cell culture, ca 15 mg of pure protein was purified to homogeneity. Mass spectrometric analysis showed that the recombinant protein has exactly the expected mass, of 11,436.74 Da. The visible spectrum (data not shown) is identical with that of the cytochrome c in the intact enzyme, with maxima at 417 nm and 551 nm in the difference spectrum reduced K oxidized; however, the split of the alpha band at liquid nitrogen temperature, observed for the full enzyme, was not detected for the reduced protein. In the oxidized state, the electronic spectrum displays a band at ca 695 nm, typical of His-Met iron axial coordination. The reduction potential was determined by redox titrations monitored by visible spectroscopy, at pH 6–8.5 (Figure 1). The redox behaviour was reversible, as judged by performing the titrations in the reductive and oxidative directions and, within experimental error, the heme potential was found to be pH-independent within that range, and to have a value of 272(±3) mV, very close to that of the intact protein (260 mV).

Polypeptide chain conformation

An overall view of the crystal structure of the cytochrome c domain of caa3 HiPIP:oxygen oxidoreductase is represented in Figure 2. The secondary structure assignments were made with the program DSSP16 and the final model consists of five α-helices and two antiparallel β-strands connected by random coil segments. Three of the five helices, namely the N-terminal helix 1 (residues 5–15), the

![Figure 1. Redox titration at pH 6 of R. marinus caa3 HiPIP:oxygen oxidoreductase monitored by visible spectroscopy. The points (×, reductive direction; □, oxidative direction) represent the absorbance at the Soret maximum. The full line was calculated by a monoelectronic Nernst equation, with $E_0^{\circ} = 272$ mV.](image-url)
C-terminal helix 5 (residues 85–96) and the middle helix 3 (residues 54–62) are long and well formed. The other two short helices (residues 18–20 and residues 78–82) are 310 helices and less well formed. Two short β-strands (residues 41–43 and 49–51) are located between the random coil 1 and the middle helix 3.

Heme geometry and interactions

A representative part of the final $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the heme prosthetic group with the Fe atom is depicted in Figure 3. The heme group is almost completely buried in a hydrophobic pocket in the protein. Only few atoms in the C-ring are exposed to the solvent, the atoms CMC, CBC and CAC at the edge of the ring being the most exposed. This group is the most rigid element in the structure and is very well refined. The rigidity is provided partly by the ligands to the heme, Cys18 (bond distance SG-CAB = 1.87 Å) and Cys21 (bond distance SG-CAC = 1.87 Å), that are part of the helix 2 and the long loop 1 (residues 20–41) and covalently bonded to the C-atoms in the C-ring mentioned above. The histidine ligand, His22, is also part of the loop 1 and the ligand Met76, is part of the second loop spanning residues 62–78. The iron atom lies in the plane of the heme and its distance to the axial histidine residue is 2.02 Å and to that of the methionine residue is 2.28 Å. The Fe atom is slightly displaced towards the histidine residue, and is 0.22 Å out of the porphyrin ring plane. The pyrrole rings B and C show the greatest deviations, as they appear to be pulled by the thioether linkages with the cysteine residues.

The heme group is further held in place by a salt-bridge between the O1A atom of the propionate ring A and NH1 of Arg42 (O1A–NH1 = 2.80 Å). The oxygen atom, O1A, further forms a hydrogen bond to a water molecule with a distance of 2.86 Å. One other contact between the heme group and the polypeptide backbone is formed by a tryptophan residue hydrogen bonded to the propionic acid group of the heme in molecules of cytochrome c, c550, c2 and c551. In the cytochrome c domain of the caa₃ HiPIP:oxygen oxidoreductase this role is played by a sequentially unrelated tyrosine residue, Tyr56, which is a hydrogen bond donor to the O2A of the propionate group with a distance of 2.57 Å.

Electrostatic properties and molecular recognition

The electrostatic potential map at the molecular surface of the cytochrome c domain of caa₃ HiPIP:oxygen oxidoreductase was calculated using the program GRASP to provide insights into the binding with its electron donor HiPIP on one side and its interaction with the CuA containing, remaining subunit II, on the other side. The electrostatic potential at the molecular surface of the domain viewed from the side proximal to the heme prosthetic group is presented in Figure 4(a). The crystal structure does not show a marked electrostatic character, especially around the heme edge region, which is, in general, one of the most exposed regions for electron transfer. The residues leucine 29, valine 30 and valine 75 around the heme edge region are mostly hydrophobic and are exposed to the solvent in the crystal structure. The surface potential map reported for the homology model also suggested similar hydrophobic properties around this region. There is no crystal structure available for the redox partner HiPIP from R. marinus. But, based on the crystal structures of HiPIP from other species, it can be deduced that...
there is a hydrophobic patch on the surface adjacent to the $[4\text{Fe}–4\text{S}]^{2+/3+}$ cluster that is well conserved among different species. Therefore, it can be proposed that the interaction of the cytochrome $c$ domain of $\text{caa}_3$ HiPIP:oxygen oxidoreductase with its redox partner is hydrophobic in nature. Of course, further studies are necessary to fully understand the nature of the interaction between the cytochrome domain and its electron donor.

In contrast to the neutral nature of the electrostatic properties of the molecule viewed from the proximal side of heme, the distal side to the heme group is mostly positively charged, as shown in Figure 4(b). This is attributed to the presence of a positively charged cluster in the structure, created by lysine and arginine residues around the propionate groups of the heme. There is no crystal structure available for subunit II of the $R.\ marinus\ caa_3$ HiPIP:oxygen oxidoreductase to clearly propose a docking mechanism with the cytochrome domain. But an electrostatic surface potential map calculated for the homology model derived for the subunit II of the $R.\ marinus-caa_3$ HiPIP:oxygen oxidoreductase has been reported. A large negatively charged region can be seen at the interacting surface, as shown in Figure 4(c). This suggests that the remaining part of subunit II, the CuA, could interact with the positively charged lysine and arginine patch in the soluble domain by electrostatic complementarity.

Comparison to other cytochrome structures

Cytochromes typically consist of about 80–110 amino acid residues that possess mainly $\alpha$-helices as secondary structure elements. An exception to this has been reported previously for one cytochrome structure, namely cytochrome $c_{552}$ from $\text{Thermus thermophilus}$ and has been described as an unusual structural feature for a cytochrome. The crystal structure presented here shows an interesting property, the existence of two short antiparallel $\beta$-strands forming a small $\beta$-sheet in the lower loop region that are normally absent from other cytochrome structures.

To analyse this new variation to the classical fold for the cytochrome domain, a three-dimensional structural search was carried out with the crystal structure co-ordinates of the cytochrome $c$ domain of $\text{caat}_3$ HiPIP:oxygen oxidoreductase to that of other molecules in the PDB database by using the program DALI. A cytochrome $c$ isozyme 1 (PDB ID 1YCC) from $\text{Saccharomyces cerevisiae}$ and cytochrome $c_{551}$ from $\text{Pseudomonas auregina}$ (PDB ID
R. marinus caa₃ Cytochrome c Domain Structure

(a)

(b)

(c)

Figure 4. Electrostatic potential map at the molecular surface of the cytochrome c domain from R. marinus caa₃ HiPIP:oxygen oxidoreductase. Red zones correspond to negative potentials and blue to positive potential values. The range spans \( -10 \) to \( +10 \) \( k_B T \) (\( k_B \), Boltzmann constant and \( T \), temperature). (a) The molecule is viewed from the side proximal to the heme. (b) Viewed distal to the heme. (c) The same potential map at the molecular surface of the subunit II, the CuA of R. marinus caa₃ HiPIP:oxygen oxidoreductase. The Figure was prepared using the program GRASP.¹⁸

With RMSD values for all the C\(^{\alpha}\) atoms of 2.0 Å and 2.2 Å, respectively, were chosen for further analysis as the crystal structure of the cytochrome c domain of the HiPIP:oxygen oxidoreductase shares some similarities from both c and c₅ families of cytochromes. A superimposition of the three-dimensional C\(^{\alpha}\)-coordinates was done by a least-squares algorithm implemented in the program O²³ and is depicted in Figures 5(a) and (b). The structures align well but the most significant difference to note is in the lower loop region. As shown in Figure 5(a), the lower loop is long in the cytochrome c domain of caa₃ HiPIP:oxygen oxidoreductase crystal structure as in the cytochrome c isozyme 1 from S. cerevisiae that belongs to the cytochrome c family but the orientation is different with respect to the functional heme group in both the structures. The loop orientation in the crystal structure is clearly very different from that observed for the caa₅ family members, as seen from the superposition with the cytochrome c₅₅₁ from P. aeruginosa, illustrated in Figure 5(b). Therefore, the loop size and orientation not only provides different electrostatic properties for binding to the remaining subunit II as mentioned in the previous section but distinguishes the crystal structure from the members of c and c₅ family.

To investigate further if these distinguishing structural features of cytochrome c domain of caa₃ HiPIP:oxygen oxidoreductase from the classical cytochrome fold could be a common feature for other caa₃ oxygen oxidoreductases, the amino acid sequence of the cytochrome c domain of caa₃ HiPIP:oxygen oxidoreductase was compared with all available sequences of caa₃ terminal oxidases (Figure 6), as well as with monoheme cytochromes of all known families. Significant identities and similarities were only found for the cytochrome c domains of the terminal oxidases. With any other cytochromes, the identities are below 20%, and no particular cytochrome family appears to be more closely related to those domains, i.e. they appear to constitute a new subfamily of cytochromes, as concluded also from the analysis of the cytochrome c domain of the caa₃ HiPIP:oxygen oxidoreductase structure. Within cytochrome c domains of the caa₃ terminal oxidases, two main clusters are easily identified, that correspond to the Bacillus species (for which there is the largest number of known sequences, with identities between themselves ranging from 30% to 58%, and similarities from 44% to 76%), and that corresponding to phylogenetically distant organisms (identities between 36% and 57%, similarities from 54% to 69%). This second cluster includes several enzymes having the YS motif in subunit I, with the exception of those from Ralstonia metallidurans and Magnetospirillum magnetotacticum. The T. thermophilus caa₃ has the most distant cytochrome domain within this family of enzymes. The same type of clustering was obtained when comparing the complete subunit II, just the membrane and copper domains, or even the subunit I. Among the mostly conserved amino acid residues within the cytochrome c domains of the caa₃ terminal oxidases, and excluding the heme axial ligands and the cysteine residues providing the heme attachment, very few residues seem to have a clear functional significance. For example,
Figure 5. Superposition of the Cα-backbone trace of the crystal structure with (a) cytochrome c₅₅₁ from *P. aeruginosa*, (b) cytochrome c isozyme 1 from *S. cerevisiae*. The structures were aligned by the least-squares algorithm implemented in O. The crystal structure is shown in blue and the aligned cytochromes in orange. The heme group is illustrated in green.
tyrosine 56 establishes a hydrogen bond to the heme propionate A, but is not conserved in the \textit{Bacilli} enzymes; arginine 42, which also establishes hydrogen bonds with the same propionate, is conserved only in a few enzymes. To validate this analysis further, homology models were built for the \textit{Bacillus} and \textit{T. thermophilus} species using the crystal structure co-ordinates of \textit{R. marinus} caa$_3$ HiPIP:oxygen oxidoreductase as the template. The program Swiss-model$^{24}$ was used to derive the models. A superposition of the C$^\alpha$-backbone co-ordinates is presented in Figure 7. As seen from the Figure, all the known caa$_3$ HiPIP:oxygen oxidoreductases appear to share common structural features, forming a new subfamily of cytochromes.

**Figure 6.** Amino acid sequence alignment of the cytochrome c domains from \textit{R. marinus} caa$_3$ oxygen oxidoreductase subunit II (cession numbers in parentheses). \textit{R.}, \textit{Rhodothermus} (CAC08531), \textit{L.}, \textit{Leptospira interrogans} serovar lai str. 56601 (AAN47441), \textit{Pirellula} sp (CAD73773), M., \textit{Magnetospirillum} (ZP_00051225), G., \textit{Geobacter} (ZP_00081850), R., \textit{Ralstonia} (ZP_00024396), B., \textit{Bacillus} (firmus) (AAA22364), halodurans (BAB06334), PS3 (BAA03045), \textit{anthracis} (AAP27878), \textit{subtilis} (CAB13342), \textit{G.}, \textit{Geobacillus} (BAA11111), \textit{O.}, \textit{Oceanobacillus} (BAC13393), \textit{Th.}, \textit{Thermus} (AAA27484). Strictly conserved amino acid residues are shaded in black. Alignments were made using Clustal W version 1.6 and manually adjusted.

**Figure 7.** Superposition of the C$^\alpha$-backbone trace of the crystal structure (blue) with the homology model derived for \textit{B. subtilis} (red) and \textit{T. thermophilus} (yellow) of the cytochrome c domains of caa$_3$ HiPIP:oxygen oxidoreductases.

**Conclusions**

The first three-dimensional structure of a cytochrome c domain of caa$_3$ HiPIP:oxygen oxidoreductase was solved at 1.3 Å resolution. The structure reveals the insertion of two short antiparallel $\beta$-strands forming a small $\beta$-sheet, an interesting variation to the classical all $\alpha$-helical cytochrome c fold. This modification appears to be common to all known caa$_3$-type terminal oxidases, as judged by comparative modelling and by analyses of the available amino acid sequences for these enzymes. The electrostatic potential map at the molecular surface of this extra C-terminal domain shows that the binding to its redox partner,
HiPIP, could be hydrophobic in nature and to that of CuA, the remaining subunit II, by electrostatic complementarity. The crystal structure analysis clearly provides the basis and expands the diversity for further studies on binding properties of cytochrome c domain with the redox partner, HiPIP, and to that of CuA, the remaining subunit II.

Materials and Methods

Cloning and expression of the cytochrome c domain of subunit II of R. marinus caa3 HiPIP:oxygen oxidoreductase

Standard DNA procedures were carried out as described. Genomic DNA extracted from R. marinus cells was used in a PCR together with primers 5′AAAGTCGACATGCCGCTGGCCAGCTGGGCC CCCC3′ and 5′AAGGATCCATGGCACCTTCGGAGCG TACGC AATTACTGC-3′ (the underlined bases were modified in order to insert Sall and BamHI restriction sites, respectively) to amplify a 294 bp product, which was cloned in IPTG-inducible vector pET-12a (Novagen), previously digested with the same enzymes, using E. coli strain DH5α and sequenced, confirming it to encode the 97 amino acid residues corresponding to the cytochrome c domain of subunit II of R. marinus caa3 HiPIP:oxygen oxidoreductase. In addition to the wild-type protein, the N terminus of the recombinant protein contains two extra amino acid residues, a serine and a threonine. Recombinant pET-12a plasmid was extracted with Kit Genome (Invitrogen) and transformed into E. coli BL21DE3 Gold (Stratagene) carrying the pEC86 vector. Expression was induced with 0.5 mM IPTG, in Luria broth supplemented with 8 mg/ml of FeSO4, at 37 °C, in a 10 l reactor, with a yield of 4 g/l.

Protein purification

All chromatographic steps were performed on a Pharmacia HiLoad system, at 4 °C. E. coli cells were broken using a French press at 6000 psi, in 10 mM Tris–HCl (pH 7.6); cell debris was removed by a 15 minutes centrifugation at 20,000 g. The membrane fraction was separated by ultracentrifugation at 138,000 g, at 4 °C, for 12 hours. The soluble fraction was dialyzed against 20 mM Tris–HCl (pH 7.6), 1 mM PMSF (buffer A), for 12 hours at 4 °C, applied to a Q-Sepharose column, equilibrated in the same buffer, and was eluted with a linear gradient of 0–0.5 M NaCl, for 8 hours at 4 °C, using a 2 mℓ/hr flow rate. The fraction containing the cytochrome domain did not stick to the column and eluted with buffer A. It was then applied to a gel-filtration G-50 column, equilibrated and eluted with buffer A plus 150 mM NaCl. After all purification steps the visible spectra were monitored. The protein was judged to be pure by SDS-PAGE and mass spectrometric analysis.

Spectroscopic techniques

UV–visible absorption spectra were obtained on a Shimadzu UV-1603 spectrophotometer, at room temperature, or on an OLIS DW2 spectrophotometer equipped with a liquid nitrogen cell. The samples were diluted in 20 mM Tris–HCl (pH 8), 1 mM PMSF.

Protein and heme determination

Protein concentrations were determined using the BCA Protein Assay Kit. The heme content was determined from redox spectra using a heme molar absorption coefficient of ε = 22 × 10³ M⁻¹ cm⁻¹ at the z-band maximum.

Redox titrations

Anaerobic potentiometric titrations were followed by visible spectroscopy at 25 °C and at different pH values as described. The redox potentials are quoted in relation to the standard hydrogen electrode.

Mass spectra

For mass spectrometric analysis, the cytochrome domain was mixed with a solution of acetonitrile (50%), acetic acid (0.1%) and then injected with a flux of 100 μl h⁻¹ on an Esquire3000plus ion trap system (Bruker Daltonics, Billerica, MA). The instrument was run in positive ion polarity mode with an orthogonal electrospray ionization source (ESI). Data were acquired continuously over the range 150–2500 m/z. Mass spectral data were examined using the program Bruker Daltonics DataAnalysis 3.0.

Amino acid sequence analysis

Amino acid sequence comparisons with sequences from other organisms were performed using BLAST at NCBI and TIGR databases. Multiple sequence alignments were produced as previously described followed by manual adjustment.

Crystallization and data collection

The purified protein was concentrated to 10 mg/ml in buffer A plus 150 mM NaCl and crystallized by the hanging-drop, vapour-diffusion method against a reservoir containing 1.5 M ammonium sulphate and 0.2 M sodium acetate. Tetragonal crystals appeared at 18 °C within two weeks. For data collection, a single crystal was flash-frozen in a liquid nitrogen stream at 100 K in a cryoprotectant solution containing the reservoir solution plus 10% glycerol. Data sets from this single crystal were collected at the European Synchrotron Radiation Facility (ESRF) on beamline ID-29, Grenoble, to a resolution of 1.3 Å for the native and 2.9 Å at the “Fe” edge. All data were indexed, integrated and scaled using the HKL suite of programs, DENZO and SCALEPACK. The space group was P4₁2₁2₁ with unit cell dimensions a = b = 54.049 Å, c = 91.347 Å and α = β = γ = 90°. The asymmetric unit contains one molecule with a molecular mass of 11,400 Da resulting in an apparent Vm of 2.7 Å³/ Da and a solvent content of 54.4% (v/v). Data collection and processing statistics are listed in Table 1.

Structure determination and refinement

The crystal structure of the protein was solved using the MAD technique. Transformation from intensity data to structure factor amplitudes and anomalous differences was done with the program TRUNCATE in the CCP4 suite of programs. The anomalous difference Patterson map revealed a strong peak corresponding to the Fe atom in the asymmetric unit and the position was calculated
using the program RSPS.\textsuperscript{31} This position was then refined and phases computed using the program SHARP\textsuperscript{32} and solvent flattened with the program DM\textsuperscript{31} assuming a solvent content of 55%. Phasing statistics are listed in Table 2. The experimental electron density was very clear and well defined by visual inspection of the $F_o - F_c$ and $2F_o - F_c$ maps with the program O.\textsuperscript{23} As the resolution of the native data set extended to 1.3 Å and was 98.4% complete, automatic model building with refinement was attempted with the program ARP/WARP.\textsuperscript{39} Almost 96% of the model was built and most of the side-chain residues traced by ARP/WARP. Few amino acid residues, an acetate ion and a tris(hydroxymethyl) aminomethane molecule were manually built in using the program O.\textsuperscript{23} No electron density was observed for the two extra N-terminal amino acid residues, a serine and a threonine from the recombinant protein. During the final stages of the model building, 131 water molecules were added using ARP/WARP. The crystallographic $R$ factor was 22.6% and $R_{\text{free}}$ was 26.2%. Further refinement of the anisotropic displacement parameters was done using SHELXL\textsuperscript{97} and the final crystallographic $R_{\text{cryst}}$ and $R_{\text{free}}$ values for the complete model are 13.9% and 16.7%, respectively. The final model contains 746 protein atoms, 55 heterogen atoms and 131 water molecules. The stereochemical quality of the structure was examined with PROCHECK\textsuperscript{34} and WHAT-CHECK.\textsuperscript{35} A total of 87.8% residues occupy the most favoured regions and 12.2% in the additionally allowed regions on the Ramachandran map and no residue was in the disallowed region of the map. The refinement statistics and quality of the final model are summarized in Table 1.

### Protein Data Bank accession numbers

Crystallographic co-ordinates of the cytochrome $c$ domain from the $R$. marinus caa$_3$ HiPIP:oxygen oxido-reductase have been deposited in the Protein Data Bank accession code with 1W2L.

### Table 1. Data collection and refinement statistics

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#### Refinement statistics

| Number of protein atoms            | 746               |
| Number of heterogen atoms          | 55                |
| Number of water molecules          | 131               |
| $R$-factor (%)                     | 0.1308            |
| $R_{\text{free}}$ (%)              | 0.1676            |
| Resolution range (Å)               | 30–1.3            |
| RMS deviation from ideality (%)    | 0.014             |
| Bond lengths (Å)                   | 2.3               |

- *Values for the highest resolution shell are shown in parentheses and the shells are 1.33–1.30 Å and 2.95–2.90 Å for the native and Fe-edge data sets, respectively.*
- $R_{\text{ave}} = \Sigma(I - \langle I \rangle)/\Sigma I$, where $I$ is the intensity measurement for a given reflection and $\langle I \rangle$ is the average intensity for multiple measurements of this reflection.
- With respect to Engh and Huber parameters.\textsuperscript{38}

### Table 2. Phasing statistics

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</table>

- $R_{\text{cullis}}$ equals the lack of closure error divided by the iso–ano difference (generalized $R_{\text{cullis}}$ in SHARP).
- Phasing power equals heavy-atom structure factor divided by the root-mean-square lack of closure error (statistics from SHARP).
- FOM is the figure of merit.
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References


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