Structure and mechanism of the aberrant *ba*₃-cytochrome *c* oxidase from *Thermus thermophilus*

Tewfik Soulimane¹, Gerhard Buse,
Gleb P. Bourenkov², Hans D. Bartunik²,
Robert Huber³ and Manuel E. Than^{1,3}

Rheinisch-Westfälische Technische Hochschule Aachen, Institut für Biochemie, Pauwelsstrasse 30, D-52057 Aachen, ²Max-Planck-Arbeitsgruppen für Strukturelle Molekularbiologie, Gruppe Proteindynamik, MPG-ASMB, c/o DESY, Notkestrasse 85, D-22603 Hamburg and ³Max-Planck-Institut für Biochemie, Am Klopferspitz 18 A, 82152 Martinsried, Germany

¹Corresponding authors
e-mail: tsoulimane@post.klinikum.rwth-aachen.de or
than@biochem.mpg.de

Cytochrome *c* oxidase is a respiratory enzyme catalysing the energy-conserving reduction of molecular oxygen to water. The crystal structure of the *ba*₃-cytochrome *c* oxidase from *Thermus thermophilus* has been determined to 2.4 Å resolution using multiple anomalous dispersion (MAD) phasing and led to the discovery of a novel subunit IIa. A structure-based sequence alignment of this phylogenetically very distant oxidase with the other structurally known cytochrome oxidases leads to the identification of sequence motifs and residues that seem to be indispensable for the function of the haem copper oxidases, e.g. a new electron transfer pathway leading directly from Cu_A to Cu_B. Specific features of the *ba*₃-oxidase include an extended oxygen input channel, which leads directly to the active site, the presence of only one oxygen atom (O²⁻, OH⁻ or H₂O) as bridging ligand at the active site and the mainly hydrophobic character of the interactions that stabilize the electron transfer complex between this oxidase and its substrate cytochrome *c*. New aspects of the proton pumping mechanism could be identified.

Keywords: *ba*₃-cytochrome *c* oxidase/MAD phasing/membrane protein/*Thermus thermophilus*/X-ray structure

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal respiratory membrane protein complex in eukaryotic and many prokaryotic aerobic organisms. The enzyme catalyses the reduction of molecular oxygen to water concomitant with the oxidation of reduced cytochrome *c*. The free energy of this reaction is conserved as a transmembrane proton gradient that drives ATP synthesis. Some bacterial terminal oxidases use quinol as substrate (quinol oxidases). Due to the homology of their primary structures, both types of enzyme belong to the superfamily of haem copper oxidases (for reviews, see Ferguson-Miller and Babcock, 1996; Michel *et al.*, 1998).

Recently, the three-dimensional structures of two members of this superfamily have been determined.

(i) The crystal structure of the cytochrome *c* oxidase from *Paracoccus denitrificans* was solved at 2.8 and 2.7 Å resolution for the four and two subunit enzyme, respectively (Iwata *et al.*, 1995; Ostermeier *et al.*, 1997). (ii) The bovine cytochrome *c* oxidase was solved initially at 2.8 Å and refined later to 2.3 Å (Tsukihara *et al.*, 1995, 1996; Yoshikawa *et al.*, 1998a). In 1995, we described the crystallization of the *ba*₃-type oxidase from the thermophilic eubacterium *Thermus thermophilus* (Soulimane *et al.*, 1995). We recognized cytochrome *c*₅₅₂ as substrate of the *ba*₃-oxidase and determined its crystal structure at 1.28 Å resolution (Soulimane *et al.*, 1997; Than *et al.*, 1997). Furthermore, the three-dimensional structures of bioengineered soluble copper (Cu_A)-binding domains derived from subunit II of the *Escherichia coli* quinol oxidase (Wilmanns *et al.*, 1995) and the *T. thermophilus* *ba*₃-oxidase (Williams *et al.*, 1999) have been reported.

Two important observations within the current oxidase structures are: (i) a covalent bond between His233 and Tyr237 (*ba*₃-oxidase numbering) at the active site of the cytochrome *c* oxidase (Ostermeier *et al.*, 1997; Yoshikawa *et al.*, 1998a), which has been confirmed by protein chemical investigations (Buse *et al.*, 1999); and (ii) the structural characterization of proton pathways (Iwata *et al.*, 1995; Tsukihara *et al.*, 1996; Ostermeier *et al.*, 1997; Yoshikawa *et al.*, 1998a) called 'K-', 'D-' and 'H-' (bovine) or 'E-' pathways (*P. denitrificans*). These pathways are named according to the specific residues Lys354^P, Asp124^P and His413 (bovine) or Glu442^P (numbers alone correspond to the actual *ba*₃-oxidase structure and numbers followed by a P correspond to the numbering in the *P. denitrificans* crystal structure, unless noted otherwise). The 'K-' and 'D-' pathways were identified initially and their functional importance was supported further by extensive mutagenesis experiments (for a review, see Gennis, 1998a). The understanding of the catalytic function of cytochrome *c* oxidases, regarding oxygen reduction, proton pumping and electron transfer, has advanced significantly during the last decades. In spite of the known structural data as well as many spectroscopic and kinetic characterizations, many details are, however, still not known and are controversially discussed (Gennis, 1998b; Michel, 1998, 1999; Verkhowsky *et al.*, 1999).

The *ba*₃-type cytochrome *c* oxidase from the extremely thermophilic eubacterium *T. thermophilus* HB8 (ATCC 27634) is expressed under limited O₂ supply (Keightley *et al.*, 1995). It is known as a two subunit oxidase (Keightley *et al.*, 1995; Soulimane *et al.*, 1995). The amino acid sequence of subunit I shows a clear, but distant homology to other eubacterial members of the superfamily. This has been suggested to be the result of an early gene duplication preceding the separation of bacteria and archaea (Castresana and Saraste, 1995). The archaeal oxidases and the *ba*₃-oxidase described herein belong to

the SoxB type, while most other eubacterial oxidases are of the SoxM type. Alignments of subunit II of the *T.thermophilus* *ba*₃-oxidase with sequences from different species show another typical characteristic of SoxB-type terminal oxidases (Mattar and Engelhard, 1997). It lacks ~60 N-terminal residues including the first transmembrane-spanning helix. The A-type haem of the *ba*₃-oxidase corresponds to the haem A_s present in the SoxB-type terminal oxidase from *Sulfolobus acidocaldarius*: the farnesyl chain is replaced by a geranylgeranyl group (Lübben and Morand, 1994). The site of O₂ reduction of the *ba*₃-enzyme, the binuclear haem a₃ Cu_B centre, reveals a number of unusual features (Surerus *et al.*, 1992; Kim *et al.*, 1998; Giuffré *et al.*, 1999), including an unusual pattern of reactivity towards exogenous haem a₃ ligands such as CN⁻, CO, NO or H₂O₂. Cyanide binds to the ferrous but not to ferric haem a₃ of the *ba*₃-oxidase, while ligation to the ferric state is much stronger in other oxidases. The CO affinity of Cu_B in the reduced *ba*₃-oxidase may be 50–100 times higher than in the bovine heart enzyme. Silietzky *et al.* (1999) showed that the ferric *ba*₃-enzyme does not react with H₂O₂ and they postulated that the binuclear centre of the oxidized enzyme may be closed and opens after one electron reduction.

The two structurally known cytochrome *c* oxidases are very similar in sequence (>50% identity) and structure with respect to their main subunits I and II. Interestingly, the *ba*₃-oxidase shows very little sequence homology (<20% identity; Keightley *et al.*, 1995) and it lacks most of the highly conserved amino acid residues that form the proton pathways in the other oxidases. Nevertheless, the enzyme generates an electric transmembrane potential under steady-state conditions and pumps protons. While 1.0 H⁺/e⁻ is consumed, as is usual, for water formation, the *ba*₃-oxidase shows a reduced H⁺-pumping efficiency of 0.4–0.5 H⁺/e⁻ instead of ~1 H⁺/e⁻ found for the *P.denitrificans* oxidase (Kannt *et al.*, 1998).

Considering the low sequence homology as well as the different kinetic characteristics of the *ba*₃-oxidase, the structural analysis of this oxidase and its comparison with other cytochrome oxidases will help us to understand further the general function and mechanistic details of the terminal haem copper oxidases. Specific features that are present only in this oxidase may elucidate the structural requirements for its operation at high temperatures and at low oxygen concentration.

We present here the structure determination of the *ba*₃-type cytochrome *c* oxidase from *T.thermophilus* at 2.4 Å resolution, the first intrinsic membrane protein to be solved by multiple anomalous dispersion (MAD) phasing. The structure reveals the presence of a novel subunit IIa spanning the membrane. The model of this oxidase is analysed and compared with the *P.denitrificans* and bovine aa₃-cytochrome oxidase structures, showing: (i) modified proton pumping pathways; (ii) one oxygen atom (μ-oxo species, hydroxide ion or water molecule) as the bridging ligand between Cu_B and haem a₃; (iii) the exchange of the highly conserved Glu278^P to Ile235 close to the haem a₃ Cu_B centre, which optimizes the O₂ input channel; (iv) an alternative electron transfer path leading directly from Cu_A to Cu_B; and (v) that the interaction of the *ba*₃-oxidase with its substrate cytochrome *c*₅₅₂ is based mainly on hydrophobic interactions.

Results and discussion

Overall structure

An overall view of the *T.thermophilus* *ba*₃-cytochrome oxidase complex (84 884 Da, 764 residues) parallel to the membrane is presented in Figure 1. The final model is composed of three protein subunits, I, II and IIa, and forms a bundle of 15 transmembrane helices (54 × 50 Å) as well as a small periplasmic domain (35 × 29 × 20 Å). The main part of the complex is formed by subunit I with 13 transmembrane helices, which binds the haems b and a₃ as well as Cu_B. It is associated with one transmembrane helix each of subunit II and subunit IIa. Except for the N-terminal 13 residues and residues 495–501 of subunit I, the two N-terminal residues of subunit II, one N-terminal residue of subunit IIa and very few solvent-exposed side chains, all amino acids have well-defined electron density. All N-termini of the three subunits are on the cytoplasmic (negative) side of the membrane and all C-termini are on the periplasmic side. The periplasmic domain of subunit II contains the primary electron acceptor, Cu_A. Haem b and haem a₃ are located in the hydrophobic core of subunit I ~15 Å from the periplasmic surface. The distances from the Cu_A atom CU1 (located closer to the membrane) to the haem b Fe, haem a₃ Fe and Cu_B are 19.0, 21.8 and 21.6 Å, respectively. The distance between the haem b Fe and haem a₃ Fe is 13.9 Å. The Cu_B is located 4.4 Å away from haem a₃ Fe. The structure also shows the presence of three nonyl-β-D-glucoside detergent molecules. No phospholipid molecules and no additional metal ions have been seen in the structure as expected from biochemical analyses. No P, Mg, Ca or Zn could be detected using IPC-AES (data not shown).

Subunit I

The 13th transmembrane helix of subunit I is an unusual property in the oxidase superfamily; subunit I consists of only 12 transmembrane helices in most other haem copper oxidases. This 13th helix does not superimpose with any of the 22 transmembrane helices of *P.denitrificans* or the 28 α-helices of the bovine heart oxidase. The loops connecting the 13 helices of subunit I are shorter than in the other cytochrome oxidases. This is a typical property of thermostable proteins (Adams and Kelly, 1998). Edman degradation of the crystals shows that the gene-deduced N-terminal sequence (MAVRASEIS) is missing from the crystals. The following four N-terminal residues (RVYE) are disordered and not visible in the electron density map. The previously described pores A, B and C (Iwata *et al.*, 1995) can be identified if the molecule is viewed from the periplasmic side. Pore A is blocked by several aromatic residues, pore B by the binuclear centre haem a₃ Cu_B and pore C by haem b. The C-terminal part (551–560) of subunit I wraps around the top of this subunit and is located at the edge of the interface between subunits I and II on the top of pore A.

Haem b and haem a₃ Cu_B centre

Haem b is the simplest protohaem containing a low-spin iron with the two histidine residues His72/α2 and His386/α10 as axial ligands at distances of 2.2 Å each. The coordination sphere of the high-spin haem a₃ iron consists

Fig. 1. Stereo ribbon plot of the ba_3 -cytochrome c oxidase from *T.thermophilus* viewed parallel to the membrane. The 12 transmembrane helices of subunit I that are also present in the other oxidases are shown in cyan, and the additional transmembrane segment (helices $\alpha 12^*$ and $\alpha 13$) in dark blue. Subunit II is represented in red, and the new subunit IIa, which corresponds to the first transmembrane helix of subunit II of other oxidases, in yellow. The haem prosthetic groups are depicted as stick models in purple, and the copper atoms as green balls. This figure was created with MOLSCRIPT (Kraulis, 1991) and rendered with RASTER3D (Merritt and Murphy, 1994).

Fig. 2. Stereo representation of the binuclear centre haem a_3 CuB including the final $2F_{\text{obs}} - F_{\text{calc}}$ electron density map contoured at 1.0σ (blue). Haem a_3 , the histidine ligands and the covalently linked Tyr237 are shown as stick models in orange and green, respectively. The covalent bond between Tyr237 and His233 is well defined in the electron density. The $F_{\text{obs}} - F_{\text{calc}}$ difference electron density (contoured at 5.0σ , green) between the haem a_3 iron and CuB (cyan) is of almost spherical shape and is best interpreted as one oxygen atom (O^{2-} , OH^- or H_2O ; purple), located equidistant between the two metal atoms. This figure was prepared with MAIN (Turk, 1992).

of five protein constituents and is completed by the bridging ligand at the active site. The iron is in the plane of the haem and the distance to the axial histidine ligand (His384/ $\alpha 10$) is 3.3 Å, a considerably larger distance than described in the current oxidase structures (2.1 Å for the *P.denitrificans* and 1.9 Å for the bovine heart oxidases). This finding is in accordance with recent resonance Raman data (Kim *et al.*, 1998; Gerscher *et al.*, 1999). Haem a_3 is characterized by the presence of a formyl group at C8 and

a hydrophobic hydroxyethylgeranylgeranyl moiety instead of a hydroxyethylfarnesyl side chain on C2. For the aa_3 -oxidase from *P.denitrificans*, the hydroxyethylfarnesyl group leaves pore B and penetrates into the lipid bilayer, while in the ba_3 -oxidase structure the geranylgeranyl side chain is straight and reaches the cytoplasmic side, but it does not interfere with the proton pathways.

Figure 2 shows the final model and the $2F_{\text{obs}} - F_{\text{calc}}$ and $F_{\text{obs}} - F_{\text{calc}}$ electron density maps in the vicinity of the

binuclear haem *a*₃₃ Cu_B centre. The electron density of the three histidine ligands to Cu_B, His283/α7, His282/α7 and His233/α6, as well as the covalent bridge between the imidazole Nε2 (His233/α6) and the Cε2 of the phenol ring (Tyr237/α6) is well defined. The distance between the haem *a*₃₃ Fe and the Tyr237 OH is 5.6 Å. The observed $F_{\text{obs}} - F_{\text{calc}}$ difference electron density between haem *a*₃₃ and Cu_B is located symmetrically between the two metals and is of almost spherical shape. It is best interpreted as one oxygen atom (μ-oxo species, hydroxide ion or water molecule). This oxygen atom is at a distance of 2.3 Å from each of the two metals in the final refined structure.

In the bovine structure (Yoshikawa *et al.*, 1998a), the bridging ligand was interpreted as an O–O for the resting state of the enzyme, while in the case of the *P.denitrificans* structure two ligands, one water molecule at the haem *a*₃ Fe and one hydroxide ion at the Cu_B within hydrogen bonding distance, were favoured (Ostermeier *et al.*, 1997). The identification of the correct ligation at the oxygen-binding site in the oxidized state of the enzyme is important for the elucidation of the proton pumping mechanism (Michel, 1998). We have tested these models in the *ba*₃-oxidase and found that the introduction of either an O–O moiety or two ligands at this site leads to a significantly poorer fit to the observed electron density, with very high *B*-factors for the second oxygen atom (33.4 Å² for the one oxygen species; 34.6 and 51.3 Å² for the O–O model; and 31.7 and 58.1 Å² for the H₂O/OH[−] model). Recently published electrochemically induced UV/VIS difference spectra (Hellwig *et al.*, 1999) suggest that no peroxy ligand is bound in the oxidized (as prepared) state of the *ba*₃-oxidase.

Subunit II, cytochrome *c*-binding site, Cu_A centre and the novel subunit IIa

Subunit II has only one transmembrane helix (residues 4–38), which is firmly bound to subunit I. It is the only subunit with a polar domain, which consists of a 10-stranded β-barrel (β1 71–75, β2 78–86, β3 89–91, β4 95–97, β5 102–108, β6 114–118, β7 124–127, β8 133–138, β9 143–148 and β10 161–166). This domain is located at the periplasmic side of the membrane and shows similarities to the class I copper proteins, but with a binuclear Cu_A site. The membrane-spanning helix α1 and the first β-sheet are connected by two short helices (α2 and α3, Figure 3) and a long loop. In the structure of the engineered soluble domain (Williams *et al.*, 1999), this so-called 'linker region' is located far from the transmembrane part as deduced from a comparison with other oxidase structures. The authors suggest that the structure of this linker region is influenced by crystal lattice forces (Williams *et al.*, 1999). The current structure provides the native conformation of both the transmembrane helix of subunit II and this linker region.

The Cu_A site is symmetrical with respect to the two histidine (His114 and His157) and the two cysteine (Cys149 and Cys153) ligands. The distance between the two copper atoms is 2.4 Å. The imidazole rings are not co-planar as described for the engineered soluble Cu_A domain (Williams *et al.*, 1999), but the angle between these two planes is smaller than in other cytochrome *c* oxidases (~25°). The two thiolate groups of Cys149 and Cys153 act as bridging ligands between the copper atoms.

Cu2 of the Cu_A centre is coordinated by Cys149, Cys153, His114 and Met160, with distances of 2.4, 2.4, 2.1 and 2.5 Å, respectively. The distances are in good agreement with those found for the structure of the engineered soluble domain (Williams *et al.*, 1999). The second copper of Cu_A (Cu1) is coordinated by Cys149, Cys153, His157 and Gln151. The distances are 2.5 (2.3), 2.3 (2.3), 2.1 (1.9) and 2.8 (2.6) Å on average longer than those observed for the 1.6 Å structure of the engineered soluble domain (values given in parentheses).

Ludwig and co-workers (Witt *et al.*, 1998a,b) proposed that the highly conserved residues Trp121^P and Tyr122^P might be involved in the electron transfer from cytochrome *c* to Cu_A and that Glu126^P, Asp135^P, Asp159^P and Asp178^P are part of the potential binding site for cytochrome *c*. As shown in Figure 3, Trp121^P and Tyr122^P are replaced by Phe88 and Gly89, respectively. The aromatic ring of Phe86 occupies almost the same space as Tyr122^P and might, together with Phe88, substitute for the otherwise conserved residues. The remaining acidic amino acid residues, except for Asp111, are not conserved in the *ba*₃-oxidase, and the proposed cytochrome *c*-binding surface of the Cu_A domain contains, in contrast to the *P.denitrificans* and bovine enzymes, almost only hydrophobic residues. This corresponds to the hydrophobic character of the surface around the exposed haem edge of cytochrome *c*₅₅₂ (Than *et al.*, 1997; Figure 4). We conclude that the interactions between cytochrome *c*₅₅₂ and the *ba*₃-oxidase are mainly hydrophobic. This is in good agreement with the generally lowered stability of electrostatic interactions at higher temperatures and it is supported further by the experimental data of Giuffré *et al.* (1999) which show that, in contrast to the bovine and *P.denitrificans* oxidases, no tight electrostatic complex is formed between the *ba*₃-oxidase and its substrate cytochrome *c*₅₅₂.

Surprisingly, after placing all of the visible residues of subunits I and II into the experimental electron density, one additional transmembrane-spanning helix was found. The corresponding protein subunit was isolated and its amino acid sequence was determined by automated Edman degradation (data not shown). It consists of 34 amino acid residues, forming one transmembrane helix. This helix superimposes with the first transmembrane helix of subunit II of the two structurally known cytochrome *c* oxidases, but with opposite polarity. The presence of this transmembrane helix seems to be important for the function of cytochrome *c* oxidases. As was deduced from their sequences, the SoxB-type quinol and cytochrome *c* oxidases from *S.acidocaldarius* and *Natronobacterium pharaonis* both lack the first transmembrane helix in subunit II. They also contain an additional small polypeptide, which was termed SoxD and subunit IV for these enzymes, respectively (Lübben *et al.*, 1992; Mattar and Engelhard, 1997). The sequence of this short subunit from the *N.pharaonis* enzyme shows 38% identity to subunit IIa of the *T.thermophilus* oxidase (data not shown). There is no indication for the presence of additional subunits in the *T.thermophilus* enzyme which may correspond to either apocytochrome *b* (SoxC) of the *S.acidocaldarius* oxidase or subunit III of the *N.pharaonis* oxidase.

Fig. 3. Structure-based sequence alignment of subunits I (A) and II (B) of the *T.thermophilus* *ba*₃-oxidase with the *aa*₃-oxidases from *P.denitrificans* and bovine heart as well as the engineered soluble Cu_A-binding domain of the quinol oxidase from *E.coli*. The transmembrane helices are numbered α 1–13 for subunit I and α 1 for subunit II. Short connecting helices of subunit I are marked with an asterisk (*) and the β -sheets of subunit II are numbered β 1–10. The alignment is shown only for those residues that occupy comparable positions in space (~60%), and the quality of this structural alignment is indicated by dark and light blue bars for residues at almost identical positions (r.m.s.d. of these C α atoms 1.1 Å) and those that can be identified as being at similar positions within the structure (r.m.s.d. of these C α atoms 2.0 Å), respectively. All other residues show no structural similarity between the *T.thermophilus* *ba*₃-oxidase and other cytochrome oxidases. Strictly conserved amino acids are shown in red, and those with similar chemical characteristics in yellow. This figure was created with ALSCRIPT (Barton, 1993).

Electron transfer

The structure-based sequence alignment of subunit I (Figure 3) between the phylogenetically distant *ba*₃-oxidase and the *P.denitrificans* and bovine heart oxidases shows that functionally vital residues, such as haem and Cu ligands or the residues proposed for the electron transfer from Cu_A to the haems, are conserved. In addition to these residues, a highly conserved motif (YPPL, located between α 3 and α 4) can be discerned from the alignment. Tyr136 forms a tight hydrogen bond to Trp229, which is also conserved. The π -system of this residue shows, in turn, a parallel stacking interaction with the π -system of His283, one ligand for Cu_B. These residues might form an electron transfer pathway from Cu_A directly to Cu_B, which, except for the relatively long distance between Cu_A and Tyr136 (10.5 Å), involves several π -systems and very few strong hydrogen and coordinative bonds as well as the above-mentioned π -

stacking interaction (Figure 5). The electrons may be passed from Cu_A to the Tyr136 π -system by tunnelling effects (Moser *et al.*, 1992; Page *et al.*, 1999), which can be as fast as 10⁹/s for distances of ~10 Å, or via the Cu_A ligand Gln151. The two prolines and the leucine are probably conserved for structural reasons in order to provide the correct orientation of Tyr136. In accordance with the above-mentioned conservation of these residues, the same structural arrangement is found in the *P.denitrificans* and bovine heart oxidases, and a possible involvement in electron transfer has been suggested (Iwata *et al.*, 1995; Tsukihara *et al.*, 1996).

The current understanding of the oxygen reduction mechanism at the binuclear centre requires the input of at least one of the electrons via Cu_B (Hill, 1994; Michel, 1998). The electron transfer from Cu_A via haem *ab* to haem *a*₃ at the binuclear centre is well established (Hill, 1994), and the corresponding residues (Arg450, Arg449

Fig. 4. Solid surface representation of the electrostatic potential of the proposed cytochrome *c*-binding surface near the mixed valence Cu_A site of cytochrome *c* oxidases (left) and the corresponding front face of cytochrome *c* (right) for (A) the *T.thermophilus* *ba*₃-oxidase and its substrate cytochrome *c*₅₅₂ (Than *et al.*, 1997) and (B) the bovine heart *aa*₃-oxidase (Tsukihara *et al.*, 1996) and horse heart cytochrome *c* (Bushnell *et al.*, 1990). Colouring is according to the calculated electrostatic potential of the oxidized oxidases and the reduced cytochromes, and contoured from -40 kT/e (intense red) to 40 kT/e (intense blue). This figure was prepared with GRASP (Nicholls *et al.*, 1993).

and Phe385) are conserved in the *ba*₃-oxidase. We propose the above-described pathway via Tyr136, Trp229 and His283 as an additional electron transfer pathway which should be used for electrons that are provided from Cu_B to the catalytic oxygen intermediates. To our knowledge, such a direct electron transfer from Cu_A to Cu_B has never been discussed, and experimental data will be needed to prove its mechanistic significance. Kinetic experiments of internal electron transfer (Hill, 1994) show that electrons that are passed through haem *a*₃ are transferred via haem *alb*, not supporting a model of direct electron transfer from Cu_A to haem *a*₃, as proposed by Yoshikawa *et al.* (1998b), but not excluding the possibility of a direct electron transfer from Cu_A to Cu_B. Since the redox state of Cu_B is difficult to assess spectrophotometrically (Hill, 1994), the direct observation of internal electron transfer steps is usually limited to the other three redox centres.

Proton pathways, O₂ channel and water pool

The crystal structure of the *ba*₃-oxidase reveals three possible proton pathways, which originate at the cytoplasmic side of the membrane and consist of several polar amino acid residues and crystallographically well-defined water molecules connected by hydrogen bonding networks. In principle, they could provide for the transfer of protons from the cytoplasmic side to either the periplasmic side of the membrane or the active site. Two of these pathways have similarities to the previously identified 'K- and D-pathways' (Iwata *et al.*, 1995); however, most of the key residues are not conserved.

The first pathway is formed by residues that belong mainly to the transmembrane helices α6 and α8 (Figure 6A) and leads to the conserved active site tyrosine Tyr237 (α6), which is covalently linked to the Cu_B ligand His233. Its spatial location within the molecule is comparable with the previously described 'K-pathway'. The otherwise highly conserved residues Thr351^P and Lys354^P, which in the other oxidases, upon mutation to non-polar residues, result in either a less active or a

Fig. 5. Stereo representation of the electron transfer pathways in the *ba*₃-cytochrome *c* oxidase from *T.thermophilus*. The haems *b* and *a*₃ are shown in orange, the copper atoms in blue and the metal-ligating amino acid residues in green. The two arginine residues (Arg450 and Arg449) and Phe385, which are involved in the electron transfer from Cu_A via haem *b* to the active site haem *a*₃, Cu_B are depicted in purple. The residues that form the newly postulated electron transfer pathway leading from Cu_A to the aromatic ring system of Tyr136 and from there via a hydrogen bond and Trp229 to the Cu_B ligand His238 are represented in cyan, including the corresponding distances. This figure was prepared with MAIN (Turk, 1992).

Fig. 6. Stereo representation of the proton pathways, the O₂ channel and the water pool in the *ba*₃-cytochrome *c* oxidase from *T.thermophilus* including the C α traces of the transmembrane helices (thin black lines), the haems and metal ligands (thick black lines) as well as the metal atoms as red and blue balls for the haem iron and copper atoms, respectively. Residues that belong to subunit II are marked by an asterisk. (A) The new 'Q-pathway' leading from the cytoplasmic side of the membrane via the haem α_{53} coordinating His384 to the water pool on top of the haem propionates is represented in green, and the pathway that corresponds to the classical 'K-pathway' is shown in purple. (B) Residues of the proton pathway, which corresponds to the classical 'D-pathway', are shown in blue, Thr81 and Thr394 that may connect it to Ser391 of the 'Q-pathway' are in green and Ile235 in red. (C) Solid surface representation of the O₂ input channel (purple), which extends towards the active site including the upper part of the 'D-pathway' (blue) and Ile235 (red). The highly conserved amino acid Glu278^P (yellow), which is indispensable for the proton pumping activity of other oxidases, occupies there the same space as Ile235. This figure was prepared with MAIN (Turk, 1992).

completely inactive form (Gennis, 1998a), are replaced by Ser309 and Thr312, respectively. Other residues that are part of this hydrogen bonding network include the haem *a*₃₃ OH, Tyr244, Tyr248 and two internal water molecules, Wat38 and Wat4. The lower part of this pathway is formed by Asp517, His8 (SUII), Glu516, Glu15 (SUII) and Ser261. In spite of the generally low homology of this pathway to its counterparts in the bovine and *P.denitrificans* structures, Glu15 of subunit II is part of this hydrogen bonding network in all three structures and may be more important for the function of the oxidases than previously assumed. This finding is also in agreement with recent data of Ma *et al.* (1999).

The second pathway (Figure 6B) leads from Glu17 (α 1), via Tyr91 (α 2), Thr21 (α 1), two water molecules, Wat21 and Wat12, and a number of polar residues such as Ser109 (α 3), Gln86 (α 2), Ser155 (α 4), Thr156 (α 4) and Gln82 (α 2), to an internal cavity that is relatively close to the active site (12.6 Å) and filled with the water molecules Wat10 and Wat29. The further path of the protons is less clear. They could approach the binuclear centre either directly from this internal cavity or via Ser197 and Thr231. Alternatively, the protons may be transferred via Thr81 and Thr394 to Ser391 of the third pathway, which will be discussed below. The distance between the side chain O γ ¹ atoms of the two threonines as resolved in the crystal structure is ~8 Å, which is relatively large. However, if one considers rotation of their side chains, this distance can be reduced to ~5 Å, which is significantly shorter than the distance between either Wat10/29 or Thr231 and the active site. The spatial location of this pathway within the molecule is comparable with the previously described 'D-pathway'. However, even fewer residues are conserved within the primary structure than for the 'K-pathway'. In particular, one of the key residues, Glu278^P, is replaced in sequence and space by the hydrophobic Ile235. Even though this glutamate is indispensable for the functionality of the 'D-pathway' and the proton pumping activity in other oxidases (Gennis, 1998a), the *ba*₃-oxidase does pump protons, but with reduced efficiency (Kannt *et al.*, 1998).

The presence of Ile235 in the *ba*₃-oxidase optimizes the formation of a hydrophobic pore leading from the middle of the membrane to the binuclear centre (Figure 6C). A considerably smaller hydrophobic pore could be identified in the *P.denitrificans* structure and was discussed as an oxygen input channel (Riistama *et al.*, 1996). Since the *ba*₃-oxidase is expressed only under limited oxygen supply, in addition to the reduced solubility of gaseous oxygen in water at elevated temperatures, the evolutionary development of an optimized oxygen input channel seems appropriate. This finding is in good agreement with the primary structures of the archaeal *S.acidocaldarius* aa₃-quinol oxidase and *N.pharaonis* *ba*₃-cytochrome oxidase, where Glu278^P is replaced by valine. These organisms grow also under low oxygen tension and at high temperatures. The replacement of Glu278^P by Ile235 or valine eliminates one of the key residues discussed as being essential for proton pumping in other oxidases and may be responsible for the reduced proton pumping efficiency of the *ba*₃-oxidase. The formation of an optimized, hydrophobically coated oxygen input channel and hence the supply of the oxidase with sufficient

amounts of substrate is probably more important for the function of the enzyme under limited oxygen concentration than proton pumping.

The third proton pathway, termed the 'Q-pathway', starts at the cytoplasmic side of the membrane with Gln254 and leads via two internal water molecules, Wat44 and Wat19, Thr396, the carbonyl oxygen of Leu392, Wat66 and Ser391 to the above-mentioned junction to the 'D-pathway' close to Thr394 and Thr81 (Figure 6A). From there, it continues via Gln388, the carbonyl oxygen of Leu387, the haem *a*₃₃ ligand His384, Asn366, Asp372 and the propionate of the haem *a*₃₃ pyrole ring A to a water pool, which is located above the haem propionates. It is equivalent neither to the so-called 'H-pathway', which was described in detail for the bovine oxidase (Yoshikawa *et al.*, 1998a), nor to the similar 'E-pathway' (Pfützner *et al.*, 1998) reported for the *P.denitrificans* oxidase (Ostermeier *et al.*, 1997). The residues involved in the hydrogen bond network of the 'H- and E-pathways' are not conserved in the *ba*₃-oxidase structure. This 'Q-pathway' could either transfer protons independently or be involved in the pumping of protons that originate from the 'D-pathway'. In the latter case, such protons would be translocated to Ser391 of the 'Q-pathway' via Thr81 and Thr394. Interestingly, this pathway includes the axial haem *a*₃₃ histidine ligand His384, which shows a weaker bond to the haem *a*₃₃ iron, as compared with the other oxidase structures. One might postulate that His384 undergoes structural changes during the catalytic cycle resulting in an active participation in proton pumping. The lowered H⁺-pumping activity of the *ba*₃-oxidase may then be correlated with either a completely different H⁺-pumping mechanism including this His384 and not Glu278^P or with the differences in the ligation of haem *a*₃₃ by His384.

The described modifications at the proton pathways, i.e. the probably lowered proton uptake activity of the 'D-pathway', are in good agreement with the lowered proton pumping efficiency of the *ba*₃-oxidase (Kannt *et al.*, 1998) and a proton pumping mechanism that is based on electrostatic repulsion and charge compensation, as discussed for instance by Mitchell and Rich (1994), Rich (1995) or Michel (1998). Less efficient proton uptake from the cytoplasmic side would result in a lowered electrostatic repulsion of the protons at or near the active site and hence less efficient proton pumping. In contrast, mechanisms where the primary proton pumping step is linked directly to the redox changes at one of the redox centres and must always result in the pumping of one proton per electron transferred through this site seem less favourable. Such a mechanism should always lead to the pumping of four protons per dioxygen molecule consumed by the enzyme, which does not agree with the experimental data for the *ba*₃-oxidase. However, it cannot be excluded that multiple electron transfer pathways exist. If they are used to various extents in different oxidases, then the lowered proton pumping efficiency could be explained alternatively by these effects.

The above-mentioned water pool on top of the haem propionates is conserved among all structurally known oxidases, and many surrounding residues are either invariant or replaced by amino acids with very similar chemical properties. It is connected to the bulk solvent on

Table I. Summary of data collection

Data set ^a	Wavelength (Å)	R_{sym} (%) ^b	Completeness		Unique reflections
			Overall	Anomalous	
Remote	1.07	4.3 (14.7)	99.6	99.5	39 379
Fe-f'	1.7399	4.7 (18.6)	99.8	99.8	40 384
Fe-f''	1.7371	4.7 (29.5)	99.9	99.9	40 293
Cu-f'	1.3805	4.7 (30.5)	99.7	99.6	40 219
Cu-f''	1.3782	4.8 (29.3)	99.8	99.8	40 351

^aAll data sets were recorded from 20.0 to 2.4 Å and have a multiplicity of ~8.

^b $R_{\text{sym}} = \frac{\sum I_{\text{hi}} - \langle I_{\text{h}} \rangle}{\sum_{\text{h}} \langle I_{\text{h}} \rangle}$; values in parentheses correspond to the last resolution shell from 2.49 to 2.40 Å.

the periplasmic side of the membrane via several well-developed pathways consisting of polar amino acid side chains and additional water molecules that are hydrogen bonded. This accumulation of water molecules was also identified for the *P.denitrificans* oxidase, and its involvement in the proton exit channel(s) was supported further by mutagenesis experiments (Ostermeier *et al.*, 1997; Puustinen and Wikström, 1999). Based on the general existence of this water pool in all oxidases, its localization on top of the haem propionates and the obviously fast equilibrium with the bulk solvent, we propose it as the primary acceptor not only for pumped protons but also for the water molecules formed during the catalytic turnover of the enzyme.

Materials and methods

Purification and crystallization

The purification of *ba*₃-oxidase was described briefly by Giuffré *et al.* (1999). Further details and a protein chemical description of the terminal oxidase will be given elsewhere. For the batch crystallization, 0.5 ml of the protein solution (8 mg/ml) in 0.4% nonyl-β-D-glucoside-containing 10 mM Tris–HCl buffer pH 7.0 was transferred into a 1.5 ml Eppendorf tube, adjusted to 6% polyethylene glycol 2000 as precipitant, and sealed. Crystals appear after 5 days. Crystals were also grown by vapour diffusion in sitting drops containing 6 µl of protein solution and 2.5 µl from the 1 ml reservoir solution containing 14% polyethylene glycol 2000 as precipitant in 20 mM Bis–Tris buffer pH 7.0 at 20°C. Both crystallization methods led to crystals of about equal quality, but of different size and form (data not shown). They were harvested after 1 week, mounted in 1 mm glass capillaries and analysed using X-ray rotation photography. The crystals were 0.3–2 mm in size and belong to the space group *P*₄₁/3₂/2. The cell constants changed during data collection at room temperature (*a* = *b* = 112–116 Å, *c* = 174–190 Å). Hence, the finding of a cryo condition was indispensable.

Cryogenic data collection and structure determination

X-ray diffraction data for MAD phasing were collected at the wiggler beamline BW6 at DORIS (DESY Hamburg, Germany). In a first step, the crystal was mounted in a standard cryo-loop using paraffin oil as cryoprotectant without freezing. The crystal lattice undergoes a transformation due to evaporation of solvent through the oil layer. This transformation was followed via diffraction images. The crystal was frozen after the quality of the images had reached its optimum, thereby preserving the system in this optimal state. This procedure led to an increase in diffraction power from initially ~3 Å to 2.1 Å, and a decrease in cell constants to *a* = *b* = 112.11 Å, *c* = 161.41 Å. The crystals contain one molecule of *ba*₃-oxidase per asymmetric unit. This results in a Matthews coefficient (Matthews, 1968) of 2.98 Å³/Da, which corresponds to a solvent content of 59% in their transformed state (native crystals: 3.50 Å³/Da, 65% solvent). With a complete data set, it was not possible to solve the structure applying Patterson search techniques employing the

Table II. Summary of refinement statistics and final stereochemistry

Resolution (Å)	20.0–2.4
No. of non-H protein atoms	5851
No. of heterogen atoms	174
No. of water molecules	119
R_{cryst} ^{a,b} (%)	22.2 (25.4)
R_{free} ^{a,c} (%)	26.4 (29.9)
Average <i>B</i> (Å) ²	50.6
R.m.s. deviation bond length (Å)	0.008
R.m.s. deviation bond angles (°)	1.6
R.m.s. deviation bonded <i>B</i> -factors (Å) ²	3.853

^aValues in parentheses correspond to the last resolution shell from 2.49 to 2.40 Å.

^b $R_{\text{cryst}} = \frac{\sum |F_{\text{oh}} - F_{\text{ch}}|}{\sum F_{\text{oh}}}$ where F_{oh} and F_{ch} are the observed and the

calculated structure factor amplitudes for reflection *h*.

^c R_{free} was calculated by randomly omitting 5% of the observed reflections from refinement and *R*-factor calculation.

two known cytochrome *c* oxidase structures (Tsukihara *et al.*, 1996; Ostermeier *et al.*, 1997).

MAD data were measured from one frozen crystal at 100 K at five different wavelengths comprising the Cu and Fe K-absorption edges as well as one remote point (Table I). Diffraction data were collected up to 2.4 Å in frames of 0.2° through a continuous range of 70° followed by another continuous range of 70° for measuring Friedel opposites in inverse beam geometry using a 130 mm MAR CCD detector (Mar Research, Hamburg, Germany). These data were processed with DENZO/SCALEPACK (Otwinowski and Minor, 1993).

Anomalous and dispersive difference Patterson syntheses were calculated from each of the MAD data sets resulting in a clear solution for one of the iron sites. Subsequent phasing and analysis of the residual maps using SHARP (de La Fortelle and Bricogne, 1997) gave rise to the second Fe and the three Cu sites as well as the correct enantiomorph (space group *P*₄₃/2₁/2). The final phases were calculated with SHARP using all five wavelengths, followed by 130 cycles of density modification with SOLOMON (CCP4, 1995) as implemented in the SHARP interface. The resulting electron density map was of exceptional quality (Figure of merit after SHARP: acentrics, 0.713; centrics, 0.609; after SOLOMON: 0.825) and most of the polypeptide chain could be built, including the side chains in the initial cycle of model building. The atomic model was built using MAIN (Turk, 1992) and refined in CNS v0.3 (Brünger *et al.*, 1998). Phase combination of MAD and model phases using SFALL (CCP4, 1995) and SIGMAA (CCP4, 1995) followed by density modification using either DM (CCP4, 1995) or SOLOMON proved useful in the determination of some less well ordered residues during the initial cycles of model building. The target parameters of Engh and Huber (1991) were used throughout the refinement. During the final refinement cycles, bulk solvent correction and overall anisotropic *B*-factor scaling were applied. Water molecules were added automatically with ARP (Lamzin and Wilson, 1997) and optimized further during the final cycles of model

inspection. The model quality is summarized in Table II and was checked with PROCHECK (Laskowski *et al.*, 1993) and SFCHECK (CCP4, 1995). The coordinates were deposited with the Protein Data Bank (PDB accession No. 1EHK).

Structural analysis

The secondary structure of the enzyme was analysed using the program DSSP (Kabsch and Sander, 1983). Single residues inside the transmembrane helices that do not show ideal helical geometry were included in the assignment of the transmembrane helices. The structure of the *ba*₃-oxidase from *T.thermophilus* was compared with that of the bacterial *aa*₃-oxidase from *P.denitrificans* (Ostermeier *et al.*, 1997) (PDB accession No. 1AR1), the mitochondrial *aa*₃-oxidase from bovine heart (Tsukihara *et al.*, 1996) (PDB accession No. 2OCC), the periplasmic, soluble fragments of the *E.coli* quinol oxidase (Wilmanns *et al.*, 1995), which was engineered to contain a Cu_A centre (PDB accession No. 1CYX) and the *ba*₃-cytochrome *c* oxidase from *T.thermophilus* (Williams *et al.*, 1999) (PDB accession No. 2CUA) by r.m.s. alignment of either the five metal atoms (for comparison with the other two cytochrome oxidases), the two Cu_A atoms and their directly liganding atoms (quinol oxidase) or the Cu_A atoms (soluble fragment of the *ba*₃-oxidase).

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