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High resolution crystal structure of *Paracoccus denitrificans* cytochrome *c* oxidase: New insights into the active site and the proton transfer pathways

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ABSTRACT

The structure of the two-subunit cytochrome *c* oxidase from *Paracoccus denitrificans* has been refined using X-ray cryodata to 2.25 Å resolution in order to gain further insights into its mechanism of action. The refined structural model shows a number of new features including many additional solvent and detergent molecules. The electron density bridging the heme a_3 iron and Cu_B of the active site is fitted best by a peroxo-group or a chloride ion. Two waters or OH^- groups do not fit, one water (or OH^-) does not provide sufficient electron density. The analysis of crystals of cytochrome *c* oxidase isolated in the presence of bromide instead of chloride appears to exclude chloride as the bridging ligand. In the D-pathway a hydrogen bonded chain of six water molecules connects Asn131 and Glu278, but the access for protons to this water chain is blocked by Asn113, Asn131 and Asn199. The K-pathway contains two firmly bound water molecules, an additional water chain seems to form its entrance. Above the hemes a cluster of 13 water molecules is observed which potentially form multiple exit pathways for pumped protons. The hydrogen bond pattern excludes that the Cu_B ligand His326 is present in the imidazolate form.

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1. Introduction

Cytochrome *c* oxidase (CcO) is the terminal enzyme of the respiratory chains of mitochondria and of many aerobic prokaryotes. It uses electrons from cytochrome *c* to reduce molecular oxygen (dioxygen) to water and couples this reaction to the pumping of four protons across the membrane (for reviews see [1–3]). The different origin of the four protons consumed in water formation (from the cytoplasmic side of prokaryotes or from the mitochondrial matrix) and that of the electrons (from the external side), as well as the pumping of four protons generates a difference of the electrochemical potential of protons. This electrochemical potential gradient across the membrane is used by the adenosine 5′-triphosphate synthase by coupling the backflow of protons to the generation of ATP from ADP and inorganic phosphate.

The structures of three "canonical" CcOs, namely from the soil bacterium *Paracoccus* (*P.*) *denitrificans* [4,5], from bovine heart mitochondria [6,7] and from the purple bacterium *Rhodobacter* (*Rb.*) *sphaeroides* [8,9] have been determined by X-ray crystallography. Subunit II of CcO binds the Cu_A centre, which is reduced by cytochrome *c* from the external, periplasmic surface. Subsequently the electron is transferred to heme *a*, and then to the heme a_3/Cu_B binuclear site. Molecular oxygen (dioxygen) is supposed to bind to the heme a_3 iron, when both the heme iron and Cu_B are reduced after transfer of a second electron. Heme *a*, heme a_3 and Cu_B are located in the hydrophobic interior of the enzyme at the same height, but closer to the external surface than to the internal surface (see also Fig. 1). The protons required for water formation during the catalytic cycle therefore have to travel through more than half of the membrane. Based on the X-ray crystallographic structure determinations and on prior mutagenesis experiments [10,11] two proton transfer pathways have been identified. The K-pathway, named after its essential lysine residue Lys354, leads straight into the active site. It appears to be used only for one or two protons during the initial reduction of the oxidized enzyme, whereas the residual six or seven protons travel through the so-called D-pathway [12]. All pumped protons appear to use this longer pathway which leads from the highly conserved aspartate Asp124 to another highly conserved residue, glutamic acid Glu278. Because also two or three of the consumed protons use the D-pathway, protons have to be transferred from Glu278 either to the propionates of heme a_3 [4] or further to His326, which has been postulated to be in the imidazolate form in the oxidized enzyme and to be the proton acceptor for protons waiting to be pumped [13,14]. The pumping itself is then caused by electrostatic repulsion from another proton which enters the binuclear site and is consumed there in the chemical reaction. Such pumping mechanisms (see e.g. [15] for discussion) are based on electrostatic repulsion in accordance with Peter Rich's electroneutrality principle [16] stating that each electron transfer into the hydrophobic interior of CcO is charge compensated by the uptake of a proton. This charge compensating proton originates from the internal surface and is not consumed in the chemical

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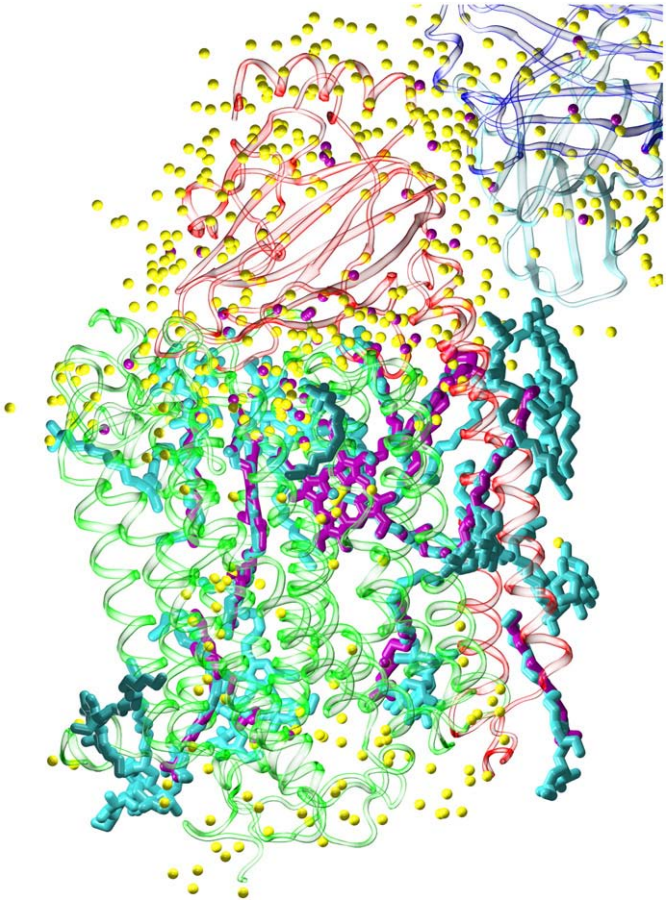

Fig. 1. Superposition of the structures obtained with data collected at 4 °C (protein data base entry 1AR1) or with cryo-cooled crystals (this work). The protein structure is shown in new cartoon representation, with subunit I colored in light green, subunit II in red and the two Fv fragment subunits in blue. Prosthetic groups and detergent molecules of the cryo-cooled structure are shown in cyan, while water molecules are colored in yellow. Non-protein atoms of the 4 °C structure are shown in magenta. Thus, in comparing yellow and magenta spheres it becomes obvious, the number of refined water molecules has increased considerably when cryo-cooling the measured crystal. The software used for visualization was VMD [54].

reactions at the active site. The active site of the fully oxidized CcO contains a Fe^{+III} (real charge +1) and a Cu^{2+} ion. Because of the close distance (4.59 Å) a negatively charged bridging ligand is required to prevent electrostatic repulsion between the two positively charged metal ions. In addition, both have unpaired electrons in their 3d orbitals, they are antiferromagnetically coupled and require a bridging ligand between each other [17].

For the bovine heart enzyme it has been published that a chloride ion might be bridging the two metal ions in the active site of the oxidized CcO, when the enzyme is purified in the presence of this anion [18]. This "resting" enzyme has to be activated by reduction, which is supposed to lead to proton uptake, and release of the chloride as HCl and replacement by an OH^- during turnover. However, in bacterial CcOs such resting states (see [19] for a review) are ill characterized or may be even absent [20].

Proton translocation in proteins is supposed to occur along hydrogen bonded chains, by shifting the proton from the hydrogen bond donor to the hydrogen bond acceptor successively along the chain. Hydrogen bonded water molecules as well as amino acid side chains can contribute to such a so-called "proton wire". For a fast and efficient proton transfer each hydrogen bond donor also has to accept a hydrogen bond at the same time. However, gaps in the hydrogen bonded chains might be bridged by reorientations of amino acid side chains with p*K* values appropriate to release and to accept protons. Examples are Glu278 in the D-pathway or Lys354 in the K-pathway.

In this publication we present the refined structure of the wild type two-subunit CcO from *P. denitrificans* complexed with an Fv fragment of a monoclonal antibody. Data could be collected using frozen crystals to a resolution of 2.25 Å, a considerable increase in resolution compared to the structure measured at 277 K [5]. Many new items could be added. In the following we put the emphasis on the results of a careful refinement of the active, binuclear site as well as of the proton transfer pathways.

2. Materials and methods

2.1. Purification and crystallization of cytochrome c oxidase

At the beginning wild type *P. denitrificans* cells were used which had been grown in a fermenter (GBF Braunschweig, kindly provided by Prof. B. Ludwig, University of Frankfurt). Crystals of CcO isolated from these cells diffracted to less than 3 Å resolution. Later crystals of CcO variants were obtained diffracting to a considerably higher resolution. Therefore "wild type" CcO was prepared using the same homologous expression system as used for the production of CcO variants. The wild type gene (isoform 2) of the *Paracoccus* CcO subunit I was cloned into the vector pUP39 [21]. The plasmid transfer into *P. denitrificans* was performed by triple mating using the *P. denitrificans* strain AO1 [22] and the *Escherichia coli* helper strain RP4-4. *P. denitrificans* wild type cells were grown in succinate medium as described [21]. The two-subunit CcO was purified via the Fv fragment 7E2 [23] and crystallized as described [5]. In order to investigate whether a suspected chloride might be present in the active site of the isolated enzyme, several CcO preparations were made using buffers where all chloride had been replaced by equimolar amounts of bromide. In addition, dithionite was added to these CcO preparations (to fully reduce CcO) and air-oxidized again three times prior to the final ion exchange chromatography step. The enzyme as isolated and crystallized has a Soret band absorption maximum at 424–425 nm which is typically for the fast or pulsed forms of CcO (see [19] for review). It is highly likely that the crystallized CcO becomes reduced during the X-ray crystallographic data collection at the synchrotron. However, due to the temperature around 100 K it is unlikely that the reduction leads to subsequent structural changes.

2.2. X-ray data collection, processing, and refinement

The crystals were cryo-protected with 25% (v/v) glycerol and flash-frozen in a stream of a gaseous cryo-stream cooled by liquid nitrogen. X-ray diffraction data were collected using synchrotron beamline X10SA (PXII) of the Swiss Light Source in Villigen, Switzerland. The two data sets collected were indexed and processed with the program XDS [24] to a resolution of 2.25 or 2.5 Å, respectively. Data collection statistics are listed in Table 1. Compared to the crystals used for collecting data at 277 K for the structure determination of CcO from *P. denitrificans* [5] (protein data base (PDB) entry 1AR1), a considerable shrinking in one unit cell dimension (*a*-axis) was observed. Therefore the structure had to be determined employing the Molecular Replacement Method using the native model 1AR1 in the program EPMR [25]. Refinement of the data against the accordingly rotated and translated 1AR1 model was started with CNS [26] employing the rigid body and annealing protocol. Later refinement was switched to Refmac5 [27], with structure factors calculated from the measured intensities using TRUNCATE of the CCP4 suite of programs [28]. Between each refinement round $2F_o–F_c$ electron density maps and $F_o–F_c$ difference-density maps were inspected utilizing the graphics program XtalView [29]. To detect firmly bound water molecules the solvent structure building routine of ARP/wARP [30] was applied. Alternatively, detergent molecules have been

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