Journal of Inorganic Biochemistry 140 (2014) 6–11

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Proton pumping by an inactive structural variant of cytochrome *c* oxidase

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ARTICLE INFO

Article history:
Received 16 April 2014
Received in revised form 23 June 2014
Accepted 23 June 2014
Available online 1 July 2014

Keywords:
Electron transfer
Membrane protein
Respiration
Electrochemical potential
Redox reaction
Cytochrome aa_3

ABSTRACT

The aa_3-type cytochrome *c* oxidases (CytcOs) from e.g. *Rhodobacter sphaeroides* and *Paracoccus denitrificans* harbor two proton-transfer pathways. The K pathway is used for proton uptake upon reduction of the CytcO, while the D pathway is used after binding of O_2 to the catalytic site. The aim of the present study was to determine whether or not CytcO in which the K pathway is blocked (by e.g. the Lys362Met replacement) is capable of pumping protons. The process can not be studied using conventional assays because the O_2-reduction activity is too low when the K pathway is blocked. Consequently, proton pumping with a blocked K pathway has not been demonstrated directly. Here, the Lys362Met and Ser299Glu structural variants were reconstituted in liposomes and allowed to (slowly) become completely reduced. Then, the reaction with O_2 was studied with μs time resolution after flash photolysis of a blocking CO ligand bound to heme a_3. The data show that with both the inactive Lys362Met and partly active Ser299Glu variants proton release occurred with the same time constants as with the wild-type oxidase, i.e. ~200 μs and ~3 ms, corresponding in time to formation of the ferryl and oxidized states, respectively. Thus, the data show that the K pathway is not required for proton pumping, suggesting that D and K pathways operate independently of each other after binding of O_2 to the catalytic site.

1. Introduction

Heme-copper oxidases are the terminal enzymes of the respiratory chain in aerobic organisms. These enzymes are membrane-bound protein complexes that catalyze the reduction of O_2 to H_2O and use part of the free energy released in this reaction for proton pumping across the membrane. In the cytochrome *c* oxidases (CytcO) the reaction involves transfer of electrons from the water-soluble electron donor, cytochrome *c*, which docks to CytcO at the more positively (*p*) side of the membrane. The primary electron acceptor of CytcO is Cu_A from where electrons are transferred to heme *a*, and then the catalytic site, composed of a heme group (heme a_3) and a copper ion (Cu_B) (Fig. 1a,b). The protons needed for reduction of O_2 to H_2O are transferred from the more negative (*n*) side of the membrane. Because electrons are transferred from the *p* side and protons from the *n* side, the O_2-reduction reaction results in a charge separation that is equivalent to moving a positive charge from the *n* to the *p* side of the membrane. In addition, part of the free energy released upon reduction of O_2 to H_2O is used for pumping of protons from the *n* to the *p* side of the membrane, thereby increasing the charge-transfer stoichiometry to two positive charges per electron transferred to O_2 (for reviews on the structure and function of the CytcOs, see e.g. [1–11]).

The heme-copper oxidases have been classified based on the architecture of the proton-transfer pathways leading from the *n*-side protein surface towards the *p*-side of the membrane as well as the catalytic site [12,13]. The well-studied bacterial CytcOs from *Rhodobacter* (*R.*) *sphaeroides* and *Paracoccus* (*P.*) *denitrificans*, belong to the A-class. In these CytcOs protons are transferred from the *n* side of the membrane through two proton-conducting pathways denoted by letters K and D, respectively (Fig. 1a). Upon reduction of the catalytic site, a net of 1–2 protons are taken up through the K pathway, while after binding of O_2 to the reduced catalytic site, the K pathway is supposedly not used, and protons are transferred though the D pathway (see e.g. [5,14–18] as well as the reviews referred to above).

Structural modifications within the D pathway typically result in slowed oxidation of the reduced CytcO due to reduced proton transfer ability. However, in some unique cases the oxidation rate as well as the rate of proton transfer through the pathway is unaffected while proton pumping is impaired (e.g. the Asn139Asp/Thr structural variants) [10,15,19–26].

Structural modifications in the K pathway result specifically in slowed reduction of the CytcO with very small effects on the reaction of the reduced CytcO with O_2. A particularly dramatic change in the CytcO reduction rate occurs upon replacement of Lys362 by Met, which yields CytcO with an O_2-reduction activity of <2% of that of the wild-type CytcO [14,27–30]. However, even though reduction of this structural variant is dramatically slowed, once the Lys362Met CytcO is (very slowly) reduced, it reacts with O_2 and becomes oxidized essentially with the same rate as the wild-type CytcO [28,31]. Data from kinetic measurements of proton transfer in this structural variant clearly showed that the slow reduction kinetics as well as turnover is caused by slowed proton transfer through the K pathway [16,28,32–34] (see Fig. 1b). The

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http://dx.doi.org/10.1016/j.jinorgbio.2014.06.016
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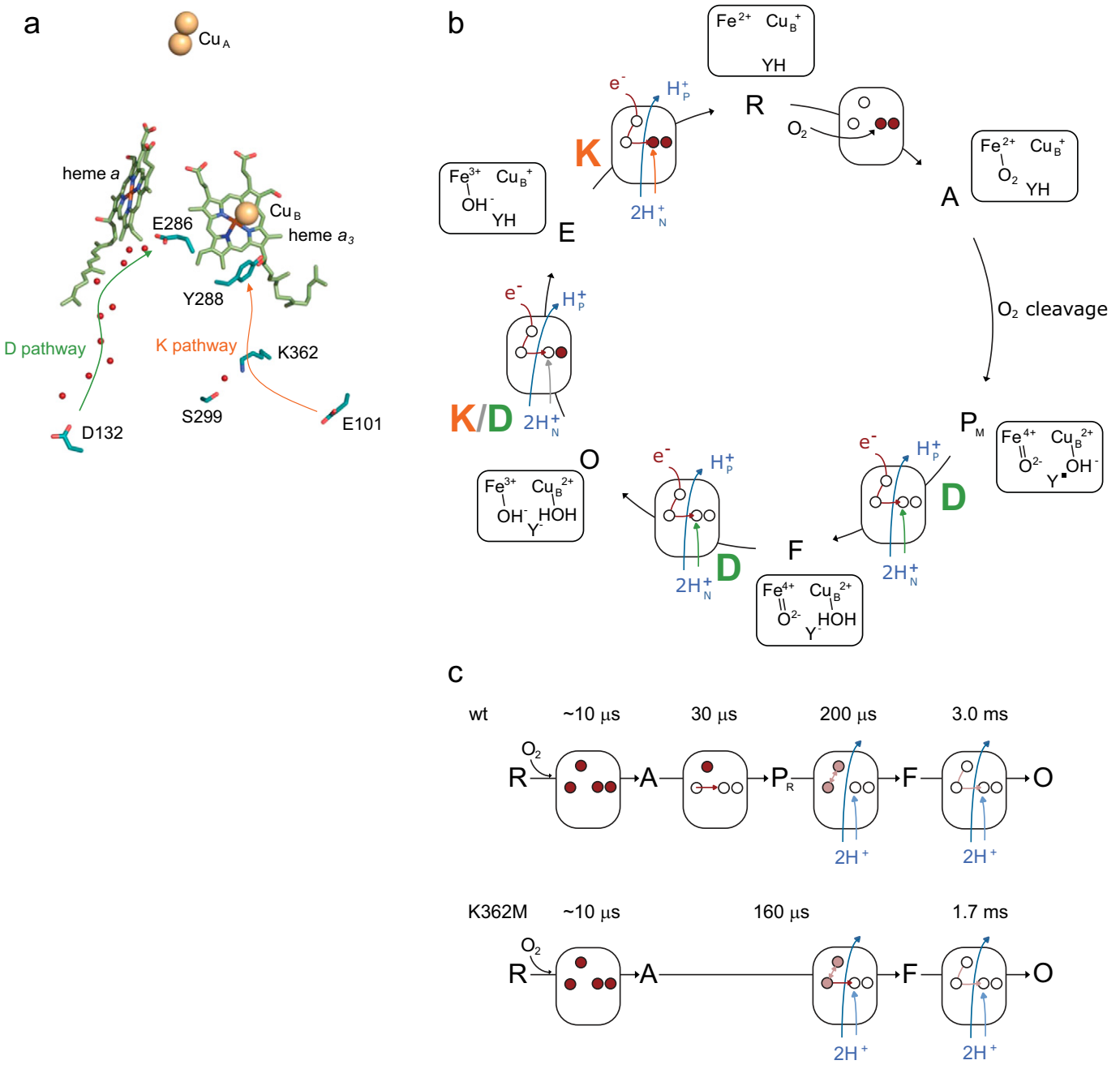

Fig. 1. The redox-active sites, the two proton pathways of the *R. sphaeroides* CytcO and the catalytic reaction. (a) The D pathway connects the *n* side of the membrane with Glu286, via water molecules (red spheres), coordinated by residues that define the proton pathway. The K-pathway starts near Glu101 in subunit II and leads via Lys362 to the catalytic site (PDB reference 1M56). (b) A schematic outline of the catalytic reaction of CytcO. Y is Tyr288 in the catalytic site. The rectangles on the arrows show the redox sites indicated as circles (Cu_A on top, heme *a* on the left and the catalytic site to the right). A filled circle indicates a reduced redox site. The rectangles near the one-letter codes indicate the structures at the catalytic site. Letters K and D indicate the pathway through which substrate protons are taken up (the "pumped protons" are presumably always taken up through pathway D). (c) A schematic outline of the reaction studied in this work with the wilde-type and Lys362Met mutant CytcOs.

conclusion that the K pathway is used for proton uptake during reduction but not oxidation of the CytcO was also supported from comparison of results from studies of the reaction of the wild-type and Lys362Met variant with H_2O_2 [35], which showed that the peroxidase activity of the Lys362Met Cyt*c*O is about the same as that of the wild-type Cyt*c*O. In this context it should be noted that even though the Lys362Met Cyt*c*O displays unaltered peroxidase-reduction activity and reacts rapidly in a single turnover with O_2, it is always referred to as *inactive* (c.f. the title of this paper) because it's (natural) O_2-reduction activity is a factor of >50 slower than that of the wild-type Cyt*c*O.

Detailed studies of the kinetics of electron and proton transfer during reaction of the reduced Lys362Met variant with O_2 showed that the rates of proton uptake were essentially the same as those measured with the wild-type CytcO [28] (Fig. 1c). Furthermore, electrogenic events, interpreted to be associated with proton uptake during oxidation of Cyt*c*O, also displayed almost the same rates with the Lys362Met variant as with the wild-type CytcO [17,36]. Consequently, investigation of the reaction of the reduced Lys362Met CytcO with O_2 can be used to study the effects of blocking the K pathway on proton pumping. Several such studies have been reported previously, all based on measurements