Bound water in the proton translocation mechanism of the haem–copper oxidases

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Abstract **We address the molecular mechanism by which the haem–copper oxidases translocate protons. Reduction of O_2 to water takes place at a haem iron–copper (Cu_B) centre, and protons enter from one side of the membrane through a 'channel' structure in the enzyme. Statistical–mechanical calculations predict bound water molecules within this channel, and mutagenesis experiments show that breaking this water structure impedes proton translocation. Hydrogen-bonded water molecules connect the channel further via a conserved glutamic acid residue to a histidine ligand of Cu_B. The glutamic acid side chain may have to move during proton transfer because proton translocation is abolished if it is forced to interact with a nearby lysine or arginine. Perturbing the Cu_B ligand structure shifts an infrared mode that may be ascribed to the O–H stretch of bound water. This is sensitive to mutations of the glutamic acid, supporting its connectivity to the histidine. These results suggest key roles of bound water, the glutamic acid and the histidine copper ligand in the mechanism of proton translocation.**

Key words: Proton translocation mechanism; Haem–copper oxidase

1. Introduction

The respiratory haem–copper oxidases catalyse the reduction of O_2 to water at a bimetallic haem iron–copper (Cu_B) centre of the enzyme. This reaction is coupled to proton translocation across the mitochondrial or bacterial membrane [1]. Protons enter the enzyme from the inside of the membrane via a proton-conducting structure (the 'D-channel'; Fig. 1) in which three amino acid residues have been shown to have key functions by site-directed mutagenesis [2–4]. The crystal structures of the haem–copper oxidases from *Paracoccus denitrificans* [5] and bovine heart mitochondria [6,7] support the presence of such a proton-conducting channel and define its structure. Some bound solvent molecules were discerned in it [5,6], but better resolution is required for a more detailed picture. Here we employ the statistical–mechanical 'potential of mean force' (PMF) methodology, which has proven a reliable tool in predicting the hydration of internal cavities and surfaces of protein crystal structures [8–10]. This approach finds several bound water molecules within the D-channel. We show that proton conduction in the D-channel can be broken by inserting a bulky side chain that breaks the connectivity of the water structure. Three water molecules connect the D-channel to the conserved glutamic acid residue 242, and at least three more water molecules are predicted by the PMF approach to link Glu^{242} further to one of the Cu_B histidine ligands (Fig. 1). However, the latter connectivity would require movement of the glutamic acid side chain. Here we present evidence supportive of such movement, and FTIR data that support the connectivity between Glu^{242} and the Cu_B histidine ligand. These results are of interest with regard to the 'histidine cycle' model of proton translocation [11].

2. Materials and methods

Escherichia coli was cultured and wild-type and mutant cytochrome bo_3 enzyme isolated and purified as described previously [12,13]. Site-directed mutagenesis was performed as described in [14,15]. All used plasmids contained a construct for a histidine tag to facilitate isolation of the enzyme in a single step [12,16]. Cytochrome bo_3 was reconstituted into liposomes as described recently [17]. Proton translocation in proteoliposomes incorporated with either wild-type or mutant enzyme was measured by the O_2 pulse method [2,3]. The methodology for obtaining FTIR difference spectra at 80 K was recently described in detail [18]. Reference spectra of the reduced carbon monoxide-treated enzyme were collected in the dark, and subtracted from 'light' spectra obtained after flash-photolysis of the sample. 'Potentials-of-mean-force' (PMF) calculations were performed as described [8–10], based on the crystal coordinates of the mitochondrial cytochrome aa_3 from beef heart mitochondria [7]. The obtained water configurations were extensively energy-minimised to a root-mean-square of the forces of $< 10^{-1}$ kcal/mol·Å. Only water molecules and polar hydrogens on the protein were allowed to move during this process.

3. Results and discussion

Fig. 2 shows that the PMF approach, in conjunction with energy minimisation, finds several bound water molecules that bridge residues[1] Asp^{91}, Asn^{80}, Asn^{98}, Ser^{101}, and Ser^{157}, in a hydrogen-bonded proton-conducting structure (the 'D-channel') of subunit I, which leads from the proton uptake side about half-way into the membrane, and ends near Ser^{157} [5,6]. This pathway is likely to be involved in proton translocation because non-conservative mutations of the first-mentioned

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Abbreviations: FTIR, Fourier transform infrared

[1] If not indicated otherwise, the amino acid numbering in this paper refers to that of the cytochrome *c* oxidase from bovine heart mitochondria, the structure [6] of which was the basis for the PMF calculations. The coordinates of the predicted water molecules are available from the corresponding author.

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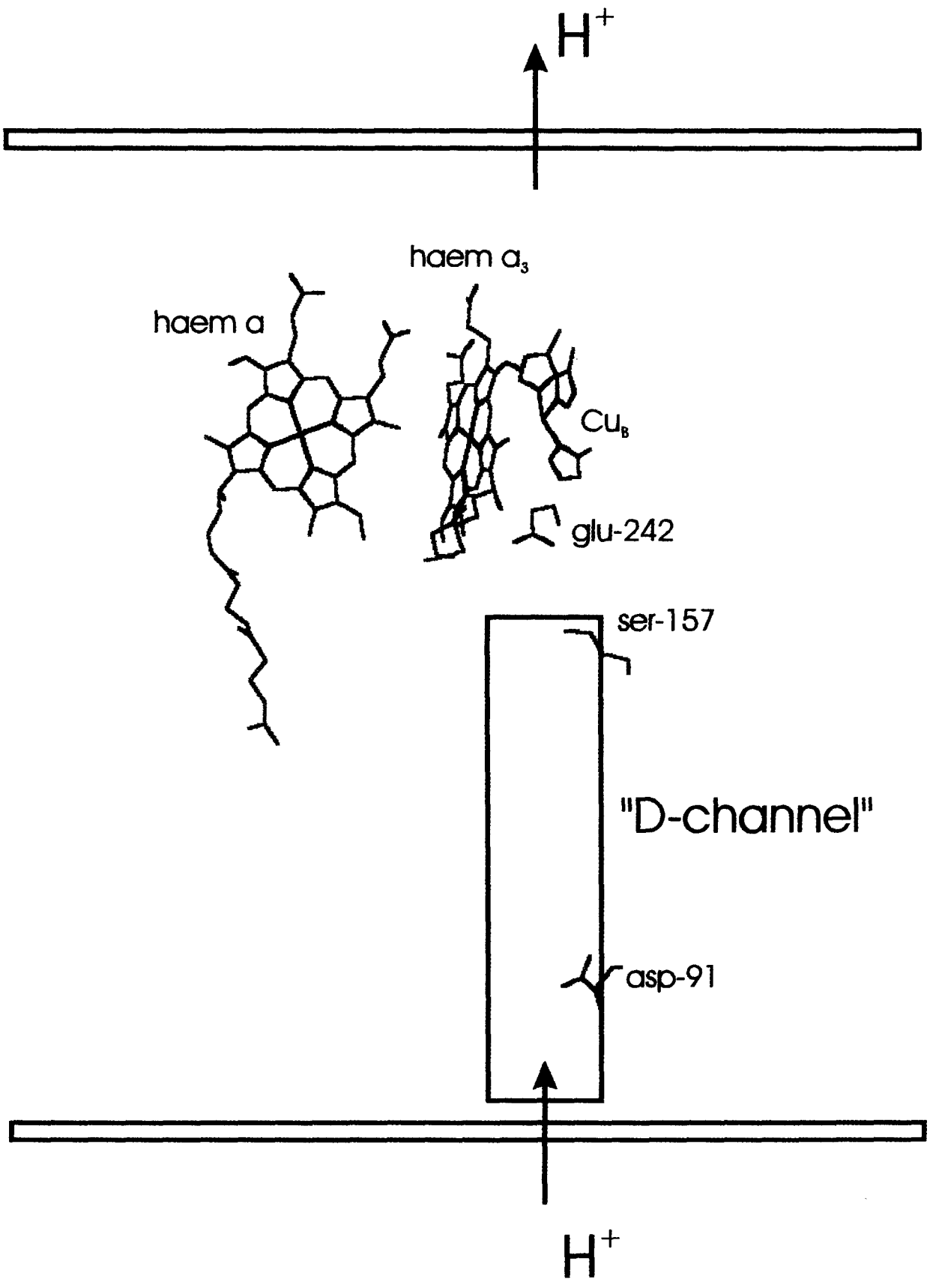

Fig. 1. Key structures in subunit I of the membrane-bound haem–copper oxidases. View in the plane of the membrane. The upper and lower bars respresent the approximate outer and inner borders of the mitochondrial or bacterial membrane, respectively. The direction of proton translocation is indicated by arrows. The rectangular structure depicts the 'D-channel' of which two key residues are indicated. A cavity intervenes between the 'D-channel' and Glu^{242}. The two haem groups and Cu_B with its three histidine ligands are also shown. All coloured structures are depicted in scale from the crystal coordinates [6].

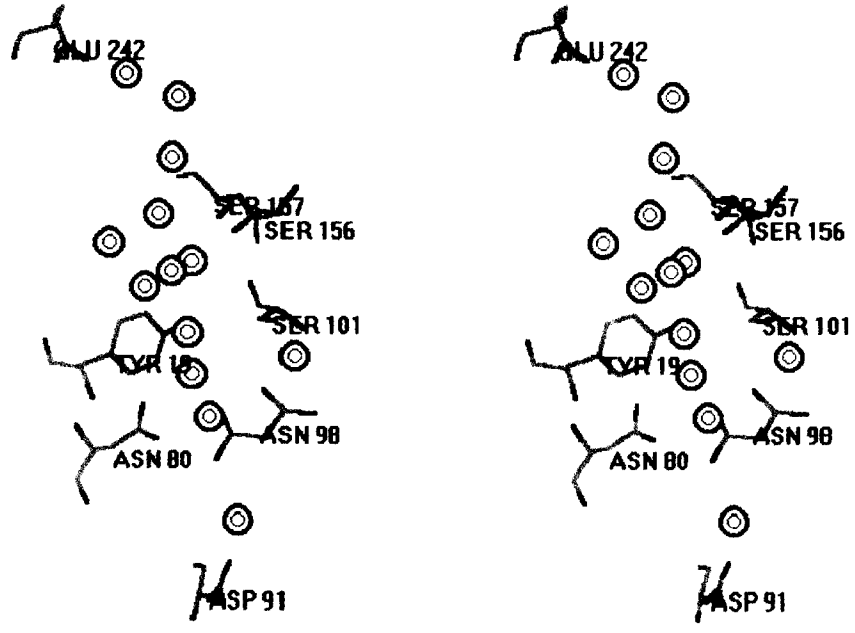

Fig. 2. The D-channel and its connection to Glu^{242}. Stereo view of a part of subunit I of the mitochondrial enzyme, that has been implicated in proton transfer. The view is approximately the same as in Fig. 1, proton translocation occurring upwards. The positions of the oxygen atoms of bound water molecules (red circles) are indicated as predicted by the PMF approach. The side chains of some of the key amino acids are also shown.

three residues have been shown to block this process [2–4]. However, mutation of Ser^{157} (Thr^{201} in cytochrome bo_3) at the top of the D-channel to alanine has no effect on the efficiency of proton translocation (H^+/e^- ratio; not shown), in agreement with the case for cytochrome aa_3 from *Rhodobacter sphaeroides* [19]. The reason for this may be that the network of bound waters near Ser^{157} is so extensive that it will not be much perturbed by the small side chain of alanine. To test this we inserted phenylalanine and tryptophan in this position. While the tryptophan mutant exhibited a normal wild type H^+/e^- ratio near 2.0, this ratio was lowered to ≈ 1 in the phenylalanine mutant, indicating significant depression of the efficiency of proton translocation (Fig. 3). Modelling these side chains into the structure (not shown) suggests that the tryptophan ring system might be orientated perpendicular to the membrane so that the water array may run aside it without much interruption. In contrast, the phenylalanine ring may be orientated parallel to the membrane, effectively interrupting the water array.

Saraste [20] and Iwata et al. [5] have suggested that an invariant glutamic acid residue in helix VI of subunit I, Glu^{242} [2], may be involved in the mechanism of proton translocation. In agreement with this, its change to cysteine was recently shown to inhibit proton translocation in the reconstituted cytochrome bo_3 enzyme from *E. coli* (where it is Glu^{286}), with much less effect on electron transfer [17]. The D-channel ends near Ser^{157} at a cavity in the middle of the membrane, and Iwata et al. [5] indicated a possible further protonic connectivity to Glu^{242} via bound water molecules. In agreement with this, the PMF approach finds three well-defined water molecules that connect the top of the D-channel to Glu^{242} by hydrogen-bonding (Fig. 4).

From Glu^{242} onwards there is no obvious protonic connectivity in the crystal structures [5,6]. However, recent FTIR data with the cytochrome bo_3 enzyme indicated a connectivity between a histidine ligand of Cu_B and the carboxylic acid group of Glu^{242}, and a hydrogen-bonded linkage through bound water molecules was suggested to provide that linkage [18]. This proposal is supported by the present PMF calculations, which predict that at least three water molecules form a hydrogen-bonded array that leads from the vicinity of the Nε of the His^{291} ligand of Cu_B towards Glu^{242} (Fig. 4). This water array does not reach the glutamic acid side chain, however, if it is orientated as in the crystal structure, but rotating it around the Cβ–Cγ bond by $\approx 180°$ puts it within hydrogen-bonding distance from the last water molecule in this array (Fig. 4). Glu^{242} is not involved in any strong interactions [6], apart from those with bound water. This suggests a functional rather than a purely structural role of this amino acid, and should facilitate side chain rotation such as that proposed here. Side chain rotation of Glu^{242} in the neutral state could be further stabilised by hydrogen bonding to the backbone oxygen of Gly^{239}.

To explore whether the side chain of Glu^{242} needs to move during proton translocation, we attempted to perturb its close vicinity in the bo_3 enzyme. The side chain of Met^{71} (Met^{116} in cytochrome bo_3) lies close to the carboxylic acid residue of the glutamic acid (the sulfur atom of Met^{71} is 3.17 Å from the nearest carboxyl oxygen of Glu^{242}; Fig. 4). Modelling changes of Met^{71} to lysine or arginine show that these side chains are likely to exhibit strong electrostatic and/or hydrogen-bonding interactions with the carboxylic acid moiety of Glu^{242}, and would hence be expected to fix its position. Both these mutant enzymes fail to translocate protons in vesicle-reconstituted cytochrome bo_3 (Fig. 3), which supports the idea that the side chain of Glu^{242} may need to move during proton translocation.

Fig. 5 shows an FTIR difference spectrum of the dissocia-

[2] The residue Glu^{242} in the mitochondrial enzyme corresponds to Glu^{278} in cytochrome aa_3 from *Paracoccus denitrificans*, and to Glu^{286} in cytochrome bo_3 from *Escherichia coli*.

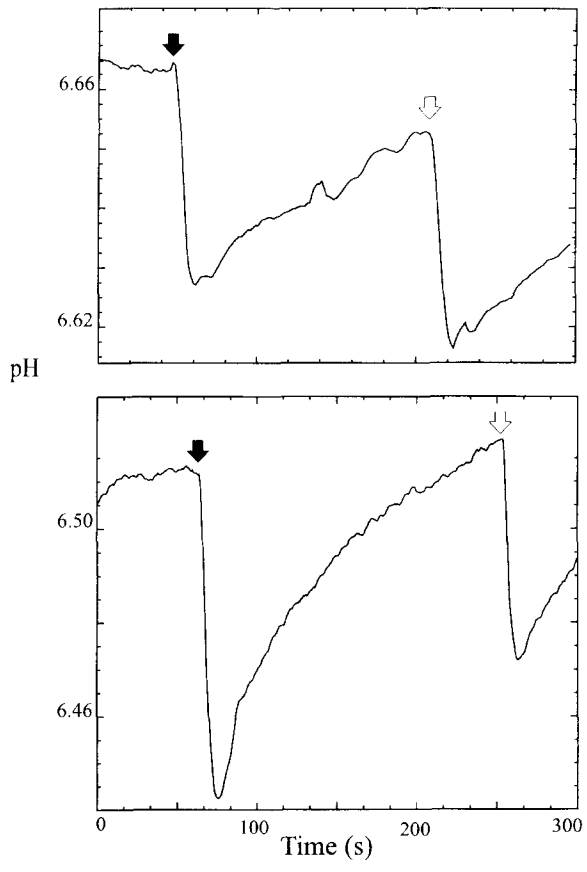

Fig. 3. Proton translocation in Thr^{201} and Met^{116} mutants of cytochrome bo_3. Proton ejection is shown from proteoliposomes into which the Met^{116}–Arg mutant enzyme (upper panel) and wild-type cytochrome bo_3 (lower panel) were incorporated. The Met^{116}–Lys and Thr^{201}–Phe mutant enzymes gave the same result as Met^{116}–Arg, while the Thr^{201}–Trp mutant showed wild-type behaviour (not shown). The anaerobic proteoliposome suspension (for methodology, see refs. [2,3,17] is pulsed with 10 μl of air-saturated water at the black arrow (= 2.58 nmol O_2, or 10.32 nequiv. of electron acceptor, at 25°). The amount of ejected protons is calibrated by injecting 10.0 nmol of anaerobic HCl (white arrow). An equal amplitude of these two responses indicates an H^+/e^- ratio of near 1.0. The H^+/e^- ratio is near 2 in the proton–translocating wild-type enzyme. The turnover in all mutants was ≈125 e^-/s, which is ≈15% of the wild-type enzyme.

tion of CO from haem iron to Cu_B by photolysis of the reduced cytochrome bo_3–CO complex in D_2O at 80 K. The trough and peak at 1960 cm^{-1} and 2065 cm^{-1} show the well-known disappearance of the C≡O bound to haem iron, and the appearance of C≡O bound to Cu_B, respectively [21,22]. We have recently shown that this perturbation shifts the carboxylic acid infrared C=O stretch of Glu^{242} in the bo_3 enzyme (where it is Glu^{286}) near 1730 cm^{-1}, suggesting connectivity between a Cu_B histidine ligand and the glutamic acid [18]. Here it is shown also to cause a 10 cm^{-1} downshift of another infrared mode near 3440 cm^{-1}. This shift, as well as that at 1730 cm^{-1} [18], is uniquely sensitive to changes at the Glu^{242} site. The 3440 cm^{-1} shift is enhanced and sharpened by changing Glu^{242} to aspartic acid, and absent or hardly visible in the corresponding cysteine mutant (Fig. 5, inset). The 3440 cm^{-1} frequency is typical for the asymmetric O–H stretch of hydrogen-bonded water [23], in which case it must be due to enzyme-bound H_2O molecules that have not exchanged with the D_2O of the medium. However, without oxygen isotope

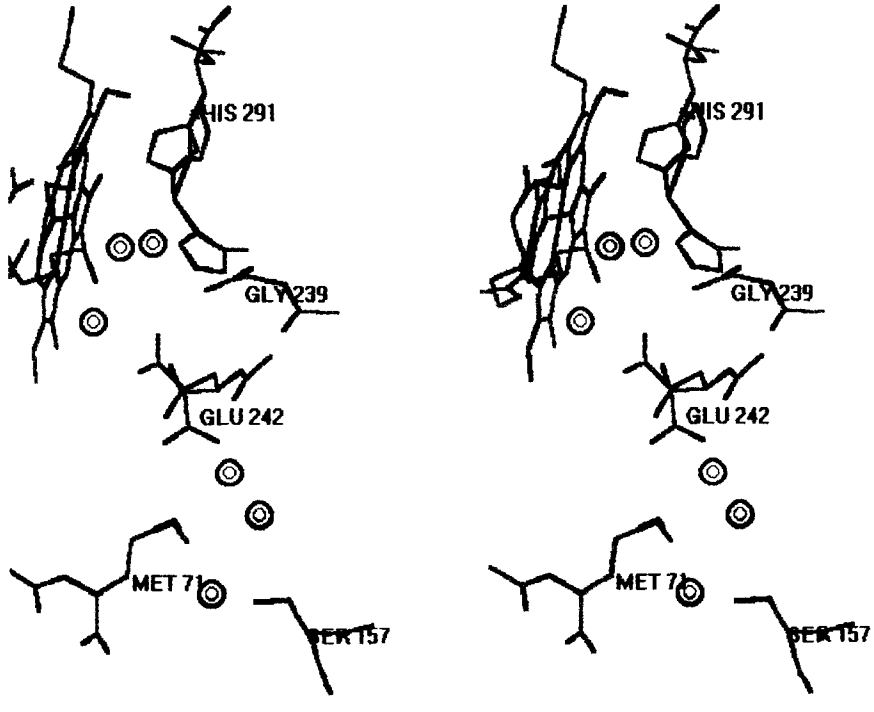

Fig. 4. Connectivity between the D-channel and the copper ligand His^{291}. Stereo view (orientation similar to Fig. 1) showing two positions of the side chain of Glu^{242} which connect it through bound water molecules (red circles), either to the upper end of the D-channel at Ser^{157}, or to the histidine Cu_B ligand His^{291}. The two other Cu_B ligands are shown unlabelled. Residues Gly^{239} and Met^{71}, as well as the haem a_3 group are also shown.

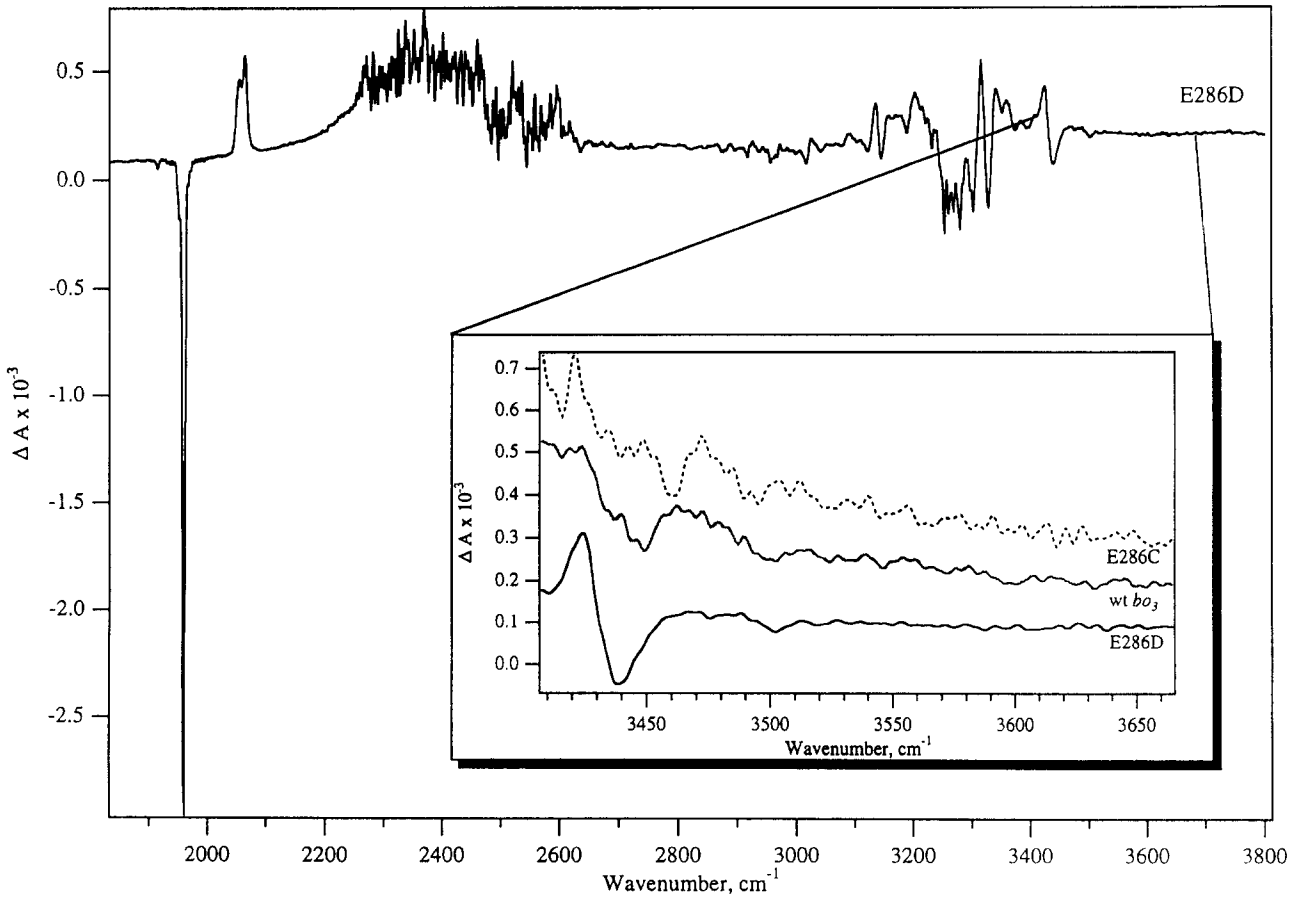

Fig. 5. 'Light' minus 'dark' Fourier transform infrared (FTIR) difference spectrum for the CO-adduct of reduced cytochrome bo_3. Enzyme samples were in 0.1 M sodium phosphate, 0.01% (w/v) *n*-dodecyl-β-maltoside buffer in heavy water (D_2O). The enzyme (0.3–0.4 mM cytochrome bo_3) was reduced with dithionite under an atmosphere of argon, and flushed with 1 atm of CO. A Perkin-Elmer 1760× FTIR spectrometer equipped with a cryostat and a mercury cadmium telluride (MCT) detector was used to record the FTIR spectra at 80 K. 'Dark' reference spectra were collected before laser photolysis, after which several 'light' spectra were recorded. Difference spectra were obtained by subtraction. The main spectrum is for the Glu^{286}–Asp mutant. The 'noisy' regions at ≈2400 and 3300 cm^{-1} are due to the high absorption of D_2O and proteinaceous N–H stretches, respectively. The inset compares wild type (wt) with the Glu^{286}–Cys and Glu^{286}–Asp mutant enzymes. These spectra are normalised to the amplitude of the haem iron-bound C≡O stretch at 1960 cm^{-1}.

data we cannot exclude that this infrared feature is due to an N–H stretch of an amine, e.g. the N–H of a histidine ligand of Cu_B. In any case, this finding provides additional strong evidence for connectivity between a histidine ligand of Cu_B and the carboxylic acid side chain of Glu^{242}. The PMF calculations reported here support the idea that this connectivity may occur through a hydrogen-bonded chain of water molecules.

Glu^{242} clearly plays an important role in the proton translocation mechanism of the haem–copper oxidases. Protons that derive from the inside of the membrane are transferred to Glu^{242} via the D-channel, which is a proton-conducting pathway that involves amino acid side chains as well as several bound water molecules (Figs. 1 and 2). The top of the D-channel (near Ser^{157}) is linked to Glu^{242} by hydrogen-bonded water molecules (Figs. 2 and 4). By a side chain rotation, Glu^{242} can make a further protonic connection to the Cu_B ligand His^{291} via bound water molecules (Fig. 4). This latter connection, which is supported by FTIR studies [18] (Fig. 5), is of particular interest in relation to the 'histidine cycle' model of proton translocation in which a histidine copper ligand was postulated to have a central switching function [5,11].

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