

Electrical current generation and proton pumping catalyzed by the *ba*₃-type cytochrome *c* oxidase from *Thermus thermophilus*

Aimo Kannt^a, Tewfik Soulimane^b, Gerhard Buse^b, Anja Becker^c, Ernst Bamberg^c,
Hartmut Michel^{a,*}

^aMax-Planck-Institut für Biophysik, Abteilung Molekulare Membranbiologie, Heinrich-Hoffmann-Strasse 7, D-60528 Frankfurt/M., Germany

^bInstitut für Biochemie, Klinikum der RWTH Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany

^cMax-Planck-Institut für Biophysik, Abteilung Biophysikalische Chemie, Kennedyallee 70, D-60596 Frankfurt/M., Germany

Received 20 July 1998

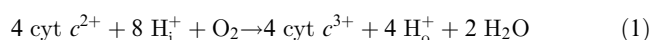
Abstract Several amino acid residues that have been shown to be essential for proton transfer in most cytochrome *c* oxidases are not conserved in the *ba*₃-type cytochrome *c* oxidase from the thermophilic eubacterium *Thermus thermophilus*. So far, it has been unclear whether the *Th. thermophilus* *ba*₃-type cytochrome *c* oxidase can nevertheless function as an electrogenic proton pump. In this study, we have combined charge translocation measurements on a lipid bilayer with two independent methods of proton pumping measurements to show that enzymatic turnover of the *Th. thermophilus* cytochrome *c* oxidase is indeed coupled to the generation of an electrocurrent and proton pumping across the membrane. In addition to a 'vectorial' consumption of 1.0 H⁺/e[−] for water formation, proton pumping with a stoichiometry of 0.4–0.5 H⁺/e[−] was observed. The implications of these findings for the mechanism of redox-coupled proton transfer in this unusual cytochrome *c* oxidase are discussed.

© 1998 Federation of European Biochemical Societies.

Key words: Cytochrome *c* oxidase; Cytochrome *ba*₃; Proton pumping; Black lipid membrane; *Thermus thermophilus*

1. Introduction

Cytochrome *c* oxidase (for recent reviews see [1,2]), as the terminal enzyme of the respiratory chains of many aerobic organisms, catalyzes oxygen reduction by cytochrome *c*, a stepwise reaction that is coupled to uptake of 1 H⁺/e[−] to form water and the translocation of 1 H⁺/e[−] across the membrane:



H_i⁺ denotes protons taken up from the inner phase (the cytoplasm), and H_o⁺ refers to protons released into the outer phase (the periplasm). The crystal structures of the *aa*₃-type cytochrome *c* oxidases from *Paracoccus denitrificans* [3,4] and bovine heart [5] have revealed two putative proton pathways that are referred to as the D- and K-pathways. The latter leads from the cytoplasm to the binuclear center and involves

residues Ser-291¹, Lys-354 ('K'), Thr-351 and Tyr-280. Mutation of Lys-354 to a methionine leads to a decrease in the rate of cytochrome *c* oxidation by a factor > 50 in the *Rhodobacter sphaeroides* and *P. denitrificans* cytochrome *c* oxidases [6,7]. However, charge-translocation studies on H₂O₂-oxidized enzyme [8] and kinetic investigations of electron and proton transfer during oxidation of the fully reduced enzyme [9] have shown that the lysine is required for proton uptake coupled to reduction of the binuclear center but is not involved in proton transfer associated with later steps of the catalytic cycle.

The longer D-pathway leads from Asp-124 ('D') via a number of polar residues (Asn-113, Asn-131, Asn-199, Tyr-35, Ser-134, Ser-193) to a highly conserved glutamic acid residue, Glu-278. Asp-124 and Glu-278 were shown to be crucial for proton pumping [10,11] and for proton uptake associated with the so-called peroxy part of the catalytic cycle, the P→O transition [8,12].

An exception to the rule are quinol and cytochrome *c* oxidases from a number of thermophilic archaea such as *Sulfolobus acidocaldarius* [13] or *Acidianus ambivalens* [14] and eubacteria like *Thermus thermophilus* [15,16]. These enzymes lack conserved residues of both the D- and K-pathways, and so far it has been unclear whether or not they function as proton pumps. In the case of the *ba*₃-type cytochrome *c* oxidase from *Thermus thermophilus* many of the D-pathway residues are not conserved, some are even replaced by hydrophobic residues (Fig. 1). Asp-124 and Glu-278 are replaced by Asn and Ile, respectively. In the K-pathway, Tyr-280 is conserved while Ser-291, Lys-354 and Thr-351 are replaced by Tyr, Thr and Ser, respectively.

In the study reported here, we have investigated – by measuring charge translocation on a black lipid membrane (BLM) with adsorbed proteoliposomes – whether the *ba*₃-type cytochrome *c* oxidase from *Thermus thermophilus* can generate an electrical current across the membrane. In addition, we have used pH potentiometric and stopped-flow spectrophotometric techniques to determine whether the *Th. thermophilus* enzyme functions as a proton pump.

2. Materials and methods

2.1. Enzyme preparation

For the expression of cytochrome *c*₅₅₂ and the *ba*₃-type cytochrome *c* oxidase, *Th. thermophilus* HB8 cells were grown at 70°C in 100 l of culture medium in a stainless steel jar fermenter under restricted aeration. The cells were harvested in the early to middle exponential

*Corresponding author. Fax: (49) (69) 96769-423.
E-mail: michel@mpibp-frankfurt.mpg.de

Abbreviations: BLM, black lipid membrane; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; *P. denitrificans*, *Paracoccus denitrificans*; Rubpy, Ru^{II}(2,2'-bipyridyl)₃Cl₂; TAME, *N*-α-tosyl-L-arginyl-*O*-methyl ester; *Th. thermophilus*, *Thermus thermophilus*; 1799, 1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptane-1

¹ If not indicated otherwise, the amino acid numbering in this paper refers to that of subunit I of the *aa*₃-type cytochrome *c* oxidase from *Paracoccus denitrificans*.

growth phase and stored at -80°C . Cytochrome c_{552} was prepared as described [17]. The ba_3 -type cytochrome c oxidase was prepared following a new protocol: 100 g frozen cells were thawed in 500 ml 100 mM Tris-HCl, pH 7.6, 200 mM KCl, and 800 mg lysozyme was added. After a 60-min stirring period the suspension was centrifuged at $17\,700\times g$ (Beckman JA-10 rotor, 10000 rpm) at 4°C . The supernatant was diluted with 5 l H_2O and chromatographed on a DEAE-biogel agarose column (Biorad) in 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.6. Fractions containing cytochrome ba_3 were pooled, concentrated and rechromatographed twice on Fractogel EMDTMAE-650 (Merck); the detergent was changed to 0.05% dodecyl- β -D-maltoside. Further purification was achieved by gel filtration (Superdex 200, Pharmacia) in 50 mM Tris-HCl, pH 7.6, 0.1% dodecyl- β -D-maltoside. The purified enzyme was concentrated (Centricon 100000, Amicon), desalted by passing through a Sephadex G-25 column, shock-frozen in liquid N_2 and stored at -74°C . A more detailed account on the preparation and protein chemical characterization of the enzyme will be given elsewhere (T. Soulimane and G. Buse, to be published).

Paracoccus denitrificans cytochrome c oxidase was isolated as described [18]. Horse-heart cytochrome c was purchased from Sigma.

2.2. Preparation of the liposomes

Proteoliposomes were prepared using the cholera dialysis method [19]. For the measurements on the BLM, *E. coli* lipids (acetone-ether extract, Avanti Polar Lipids) were used at a concentration of 10 mg/ml, vesicles for proton pumping experiments were prepared using asolectin (phosphatidyl choline type II S, Sigma) which was further purified as described in [20], at a concentration of 40 mg/ml. Lipids were dried under vacuum and resuspended in either 100 mM HEPES-KOH, pH 7.3, 10 mM KCl, 2% cholera (asolectin vesicles) or 20 mM HEPES-KOH, pH 7.3, 2% cholera (*E. coli* lipids). The suspension was stirred on ice for 1–2 h under nitrogen and sonicated to clarity with a Branson sonifier at 30% duty cycle. Cytochrome c oxidase was added to a concentration of 4 μM and the mixture was dialyzed against the resuspension buffer without cholera (4 h). Subsequently, asolectin vesicles were dialyzed against two changes of 10 mM HEPES-KOH, 50 mM KCl, 50 mM sucrose (12 h each) and two changes of 50 μM HEPES-KOH, pH 7.3, 55 mM KCl, 55 mM sucrose (12 h and 4 h). Proteoliposomes prepared with *E. coli* lipids were dialyzed three times (12 h each) against 20 mM HEPES-KOH, pH 7.3.

2.3. BLM experiments

Optically black lipid membranes were formed in a thermostated teflon cell as described by Bamberg et al. [21]. Both compartments of the cell, connected to an external measuring circuit via polyacrylamide gel salt bridges and Ag/AgCl electrodes, were filled with 20 mM HEPES-KOH, pH 7.3, and cytochrome c oxidase containing vesicles prepared with *E. coli* lipids were added to a concentration of 25 nM to one of the compartments. The vesicles were allowed to adsorb to the BLM by incubation for >30 min under stirring, yielding a capacitive coupling between proteoliposomes and lipid bilayer. 80 μM $\text{Ru}^{\text{II}}(2,2'\text{-bipyridyl})_3\text{Cl}_2$ (Aldrich) and 300 μM EDTA were added to the same compartment. Light flashes of different durations were applied using a 100-W mercury lamp (Osram) filtered through heat-protecting glass and a 395-nm cutoff glass filter, resulting in excitation of Rubpy to a state with an E_m of about -1 V which transfers an electron to cytochrome c oxidase in less than 1 μs [22]. The formed $\text{Ru}(\text{III})\text{bpy}$ is then re-reduced by EDTA as a sacrificial electron donor [23]. The resulting signal was amplified, filtered and recorded with a digital oscilloscope. For stationary current measurements, the membrane was made conductive by adding the protonophore 1799 (10 μM).

2.4. Proton pumping measurements

Proton pumping was measured as the acidification of the external medium upon a pulse of reduced cytochrome c under aerobic conditions using two different methods. In the potentiometric assay, vesicles were diluted 1:2 with the last dialysis buffer and incubated in the presence of 5 μM valinomycin in a thermostated cell (20°C) equipped with a Mettler-Toledo pH electrode until a stable baseline was reached. The enzymatic reaction was initiated by a pulse of concentrated reduced cytochrome c_{552} . Before the pulse, the pH of the cytochrome c_{552} solution was carefully adjusted to that of the vesicle suspension. Uncoupling was achieved by adding 5 μM

CCCP. The change in pH was calibrated by adding known amounts of HCl.

Proton-pumping experiments using a stopped-flow spectrophotometer were performed at 25°C , essentially as described in [24]. pH changes upon mixing of reduced cytochrome c_{552} (or horse-heart cytochrome c used in the experiments with the *Paracoccus* cytochrome c oxidase) with cytochrome c oxidase containing vesicles were followed as absorbance changes of phenol red at 558.7 nm (556.6 nm when horse-heart cytochrome c was used), a wavelength that is strictly isosbestic for the respective cytochromes. Control experiments were performed to check that, either in the absence of phenol red or when oxidized cytochrome c was used, no significant changes in absorbance could be detected (see Section 3). The pH of all solutions used in the experiment was carefully adjusted to 7.30 so as not to give any absorbance changes unconnected with the enzymatic reduction. Calibration of the observed signal was done following the change in absorbance upon trypsin-catalyzed hydrolysis of *N*- α -tosyl-L-arginyl-*O*-methyl ester (TAME, Sigma) as described by Sarti et al. [25].

3. Results and discussion

Fig. 2 shows the electrocurrents generated by cytochrome c oxidase turnover in the absence (Fig. 2A) and the presence of an uncoupler (Fig. 2B). In the first case, due to the capacitive coupling between proteoliposomes and BLM, a transient current is observed that decays with time as a potential of opposite sign builds up across the BLM. In the second case, however, the BLM is made conductive by the presence of an uncoupler and thus a stationary current is observed as long as electrons are injected into the enzyme, i.e. as long as the enzyme turns over. The sign of the current is consistent with protons – or positive charges – moving from the vesicle interior outwards. When comparing the number of translocated charges with the (estimated) number of cytochrome c oxidase molecules attached to the BLM, it immediately becomes evident that the observed current cannot be due to just simple reduction of the enzyme coupled to proton uptake from the opposite side of the membrane, but must be the result of multiple turnovers of the enzyme on a short timescale. This finding clearly establishes that the reaction catalyzed by the ba_3 -type cytochrome c oxidase is electrogenic in nature, i.e. that it is coupled to the continuous net translocation of charges across the membrane. However, using this technique it cannot be discerned whether this process is solely due to the oppositely charged reactants – protons and electrons – being taken up from opposite sides of the membrane or whether – as is the case with other cytochrome c oxidases – there is an additional translocation of protons across the membrane ('proton pumping') coupled to the enzymatic reaction.

Therefore, we have performed proton translocation experiments to investigate whether *Th. thermophilus* cytochrome ba_3 is also a proton pump. If this were true, then the turnover of cytochrome c oxidase reconstituted in proteoliposomes should lead to an acidification of the external medium. We have demonstrated that *Th. thermophilus* cytochrome ba_3 is indeed a proton pump using two independent methods of measuring the pH changes upon addition of reduced cytochrome c . As shown in Fig. 3, a pulse of reduced cytochrome c_{552} leads to a pH decrease in the outer phase that can be measured with a pH electrode in a medium of very low buffer capacity. Correspondingly, alkalization is observed in the presence of a protonophore, because then protons can equilibrate between the internal and the external medium and only the net reaction, i.e. the consumption of four protons per oxygen molecule reduced (see Eq. 1) can be observed. The H^+/e^- ratio was

	1	11	21	31	*	40	50
P.d.	MADAAVHG	DHHDTRGFFT	RWFMSTNHKD	IGILYLF-TA	GIVGLISVCF	TVYMR----	M
Th.	-----	--MAVRASEI	SRVYEAYPEK	KATLYFLVLG	FLALIVGSLF	GFFQALNYGN	
		1	9	19	29	39	
	56	66	76	86	96	106	*
P.d.	ELQHPGVQYM	CLEGARLIAD	ASAECTPNGH	LWNVMITYHG	VLMFFVVIIP	ALFGGFGNYF	
Th.	VDAYPLLKR-	-----	----LLPFVQ	SYQGLTLHG	VLNAIVFTQL	FAQAIMVYLP	
	49		58	64	74	84	
	116	*	126	*	136	146	156
P.d.	MPLHIGAPDM	AFPRLNLSY	WMYVCGVALG	VASLLAPGGN	DQMGSGVGWV	LYPPLSTTEA	
Th.	ARELNMRPMM	GL---MWLSW	WMAFIGLVVA	ALPLLAN---	---EATVLYT	FYPPLK----	
	94	104	111	121	128	135	
	176	186	**	196*	206	216	226
P.d.	GYMDLAIFA	VHVSGASSIL	GAINIITTF	NMRAPGMTLF	KVPLFAWSVF	ITAWLILLSL	
Th.	GHWAFYLGAS	VFVLSTWVSI	YIVLDLWRRW	KAANPGK--V	T-PLVTYMAV	VFWLWFLA-	
	141	151	161	171	179	188	
	236	246	256	266	*	286	
P.d.	PVLGATITML	LMDRNFQTQF	FDPAGGGDPV	LYQHILWFFG	HPEVYTIILP	GFGIISHVIS	
Th.	-SLGLVLEAV	LFLLPWSFGL	VEGV---DPL	VARTLFWWTG	HPIVYFWLLP	AYAIITYILP	
	197	206	216	223	233	243	
	296	306	316	326	335	345	
P.d.	TFAKKPIFGY	LPMVLAMAAI	GILGFVVWAH	HMYTAG-MSL	TQQAYFMLAT	MTIAVP ^{TG} IK	
Th.	KQAGGKLVSD	PMARLAFLLF	LLLSTPVGFH	HQFADPGIDP	TWKMIHSLVT	LFVAVP ^{SL} MT	
	253	263	273	283	293	303	
	355	365		372	381	390	
P.d.	VFSWIATMWG	GSIEFKT---	-----	-PMLWAFGFL	FLFT-VGGVT	GVVLSQAPLD	
Th.	AFTVAASLEF	AGRLRGGRGL	FGWIRALPWD	NPAFVAPVLG	LLGFIPGGAG	GIVNASFTLD	
	313	323	333	343	353	363	
	400	410	420	430	440	447	
P.d.	RVYHDTYYV	AHFHYVMSLG	AVFGIFAGVY	YWIGKMSGRO	---YPEWAGQ	LHFWMFIGS	
Th.	YVVHNTAWVP	GHFHLQVASL	VTLTAMGSLY	WLLPNLTGKP	ISDAQRRRLG	AVVWLWFLGM	
	373	383	393	403	413	423	
	457	467	477	485	495	505	
P.d.	NLIFFPQHFL	GRQGMPPRYI	DYPV--EFAY	WNNISSIGAY	ISFASFLFFI	GIVFYTLFAG	
Th.	MIMAVGLHWA	GLLNVPRRAY	IAQVPDAYPH	AAVPMVFNVL	AGIVLLVALL	LFIYGLFSVL	
	433	443	453	463	473	483	
	515	523	533	543	552		
P.d.	--KRVNPNY	WNEHADTLEW	TLSPPPPEHT	FETLPKRED-	WDRAHAH---	-----	
Th.	LSRERKPELA	--EAPLPFAE	VISGPEDRRL	VLAMDRIGFW	FAVAAILVVL	AYGPTLVQLF	
	493	503	511	521	531	541	
P.d.	-----						
Th.	GHLNPVPGWRLW						
	551						

Fig. 1. Sequence comparison between the subunits I of the *aa*₃-type *P. denitrificans* and the *ba*₃-type *Th. thermophilus* cytochrome *c* oxidases. The alignment is part of a multi-sequence alignment of 20 different cytochrome and quinol oxidases from several species using the ClustalW algorithm without manual adjustments. Black lines mark the transmembrane helices of the *P. denitrificans* cytochrome *c* oxidase. The histidine ligands to the metal centers are shown in bold, residues suggested as part of proton-transfer pathways in the *Paracoccus* enzyme are shown in bold italics (K-pathway), D-pathway residues are marked by asterisks.

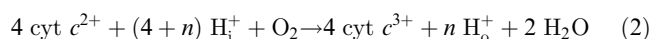
extrapolated to 0.3–0.5 for the coupled and 0.9–1.0 for the uncoupled reaction. In experiments with the *P. denitrificans* enzyme, the ratio was found to be ~ 1 for both the coupled and the uncoupled reaction (not shown). However, due to the slow response time of the pH electrode it cannot be decided whether this difference is real.

Thus, we have conducted additional, time-resolved experiments where we have followed pH changes associated with cytochrome *c* oxidation in a stopped-flow spectrophotometer. An additional advantage of this method is that, at different wavelengths, cytochrome *c* oxidation and pH changes can be monitored under the same conditions to verify that the two processes indeed correlate with each other kinetically. As shown in Fig. 4B, there is an acidification of 0.8–1.0 H⁺/e[−] after mixing reduced horse-heart cytochrome *c* with *P. denitrificans* cytochrome *c* oxidase containing proteoliposomes. It converts to an alkalization of exactly the same magnitude in the presence of an uncoupler. Following the reaction at 550

nm instead of 556.6 nm, it becomes clear that the pH changes and cytochrome *c* oxidation occur at the same rate implicating that the two processes are coupled to each other. Additionally, control experiments at 556.6 nm either with reduced cytochrome *c* in the absence of phenol red or with oxidized cytochrome *c* in the presence of phenol red showed that there were no mixing artifacts or contributions from the cytochrome *c*. Fig. 4A summarizes the same experiments done with the *Thermus ba*₃ enzyme and cytochrome *c*₅₅₂ as substrate. Again, acidification and alkalization were observed (at 558.7 nm) upon cytochrome *c* oxidation in the absence and the presence of a protonophore, respectively. The rate of the two processes was the same as that of the cytochrome *c* oxidation followed at 552.7 nm. However, whereas the H⁺/e[−] ratio was determined to be ≈ 1 for the uncoupled reaction, it was reproducibly found to be 0.4–0.5 for the coupled reaction. This can hardly be explained by proton leakage back into the interior of the vesicles, as shown by the correlation of the

rates of acidification, alkalinization (when CCCP was present) and cytochrome *c* oxidation. Additionally, *ba*₃ vesicles had an unusually high respiratory control ratio (RCR) of up to well above 50 whereas it was between 4 and 8 for the proteoliposomes with the *Paracoccus* enzyme, suggesting that the former vesicles are even less proton-permeable than the latter. Thus the difference in the H⁺/e⁻ ratio between the *Paracoccus* and the *Thermus* enzymes seems to be real.

To sum up, we have provided clear evidence that the *ba*₃-type cytochrome *c* oxidase from *Th. thermophilus* is an electrogenic proton pump that couples cytochrome *c* oxidation to the consumption of 1 H⁺/e⁻ and, at pH 7.3, the translocation of 0.4–0.5 H⁺/e⁻ across the membrane. This corresponds to a net reaction of



with *n* being ≈ 2 rather than 4 as has been described for other systems.

However, as already pointed out above, the enzyme lacks the residues that have been shown to be crucially important for proton pumping in other bacterial oxidases. Thus, our results raise the question of how the *Thermus* enzyme can translocate protons at all in the light of this low degree of sequence homology.

In the K-pathway, most of the residues (see Fig. 1) are replaced by – in terms of polarity – very similar amino acids, the exception being Lys-354 which is a threonine in the *Thermus* enzyme. Assuming the lysine to be neutral (i.e. non-protonated) in the *P. denitrificans* enzyme as has been deduced from electrostatic calculations [26], one could speculate that this pathway is still functional in the *Thermus* enzyme as it might still provide an environment polar enough to accommodate a chain of water molecules that could facilitate proton transfer to the binuclear center.

However, the situation is different for the D-pathway. Whereas some of the residues close to the cytoplasm are either conserved or replaced by similar residues, this is not the case for Asp-124 and Glu-278 that are Asn and Ile in the *Thermus*

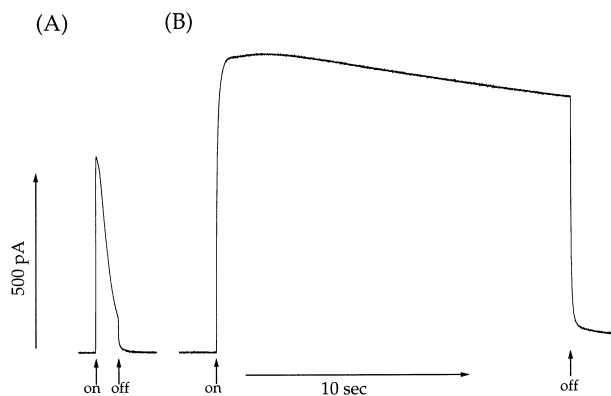


Fig. 2. Light-induced electrical currents measured with Rubpy as electron donor. *Th. thermophilus* cytochrome *c* oxidase containing vesicles (cytochrome *c* oxidase concentration 25 nM in 20 mM HEPES-KOH, pH 7.3) were attached to an optically black lipid membrane and subjected to light-flashes of various durations in the presence of 80 μM Rubpy and 300 μM EDTA. A: Capacitive current, flash duration 1 s (Hg lamp, 395 nm cutoff filter). B: Stationary current in the presence of 10 μM 1799, flash duration 16 s.

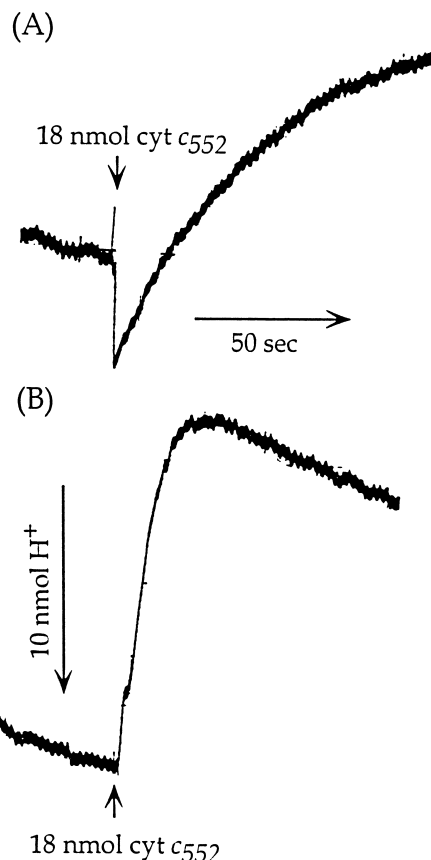


Fig. 3. A: Acidification of the external medium after addition of 18 nmol reduced cytochrome *c*₅₅₂. The reaction buffer contained 50 μM HEPES-KOH, 55 mM KCl, 55 mM sucrose, pH 7.30, and 5 μM valinomycin. The buffer in the vesicle interior was 100 mM HEPES-KOH, 10 mM KCl, pH 7.30. Proteoliposomes containing 1.5 nmol of *Th. thermophilus* *ba*₃-type cytochrome *c* oxidase were used. B: The same substrate pulse in the presence of 5 μM of the protonophore CCCP. The signals were calibrated by the addition of known amounts of HCl.

enzyme. The latter is especially surprising as both the Cu_B-ligand His-276 and the nearby Tyr-280 are conserved in the *Thermus* cytochrome *c* oxidase. The two residues have been suggested to be covalently linked to each other on the grounds of the crystal structures of the bovine [27] and *Paracoccus* enzymes [4]. Recently, it was verified using protein chemical methods that the covalent linkage exists in these two enzymes, and also in the *ba*₃- and *caa*₃-type terminal oxidases from *Th. thermophilus*, indicating it to be a common feature of heme/copper-containing terminal oxidases (G. Buse, T. Soulimane and M. Dewor, submitted). Additionally, the -GHPEVY- motif is one of the best conserved among heme-copper oxidases, and all residues of this motif but the glutamate are conserved in the *Th. thermophilus* cytochrome *ba*₃. All this indicates that the structure of the loop must be very similar in the different species. Since from the sequence alignment there also seems to be no obvious titratable residue that could be close in space (e.g. in transmembrane helix II – in fact, according to the sequence alignment, there is no titratable residue in subunit I located in the middle of a transmembrane helix) to the *Thermus* Ile-235 and thus substitute for the lack of the glutamate, we suggest that the D-pathway does not exist in the *Thermus* cytochrome *c* oxidase. Thus, as the pathway has

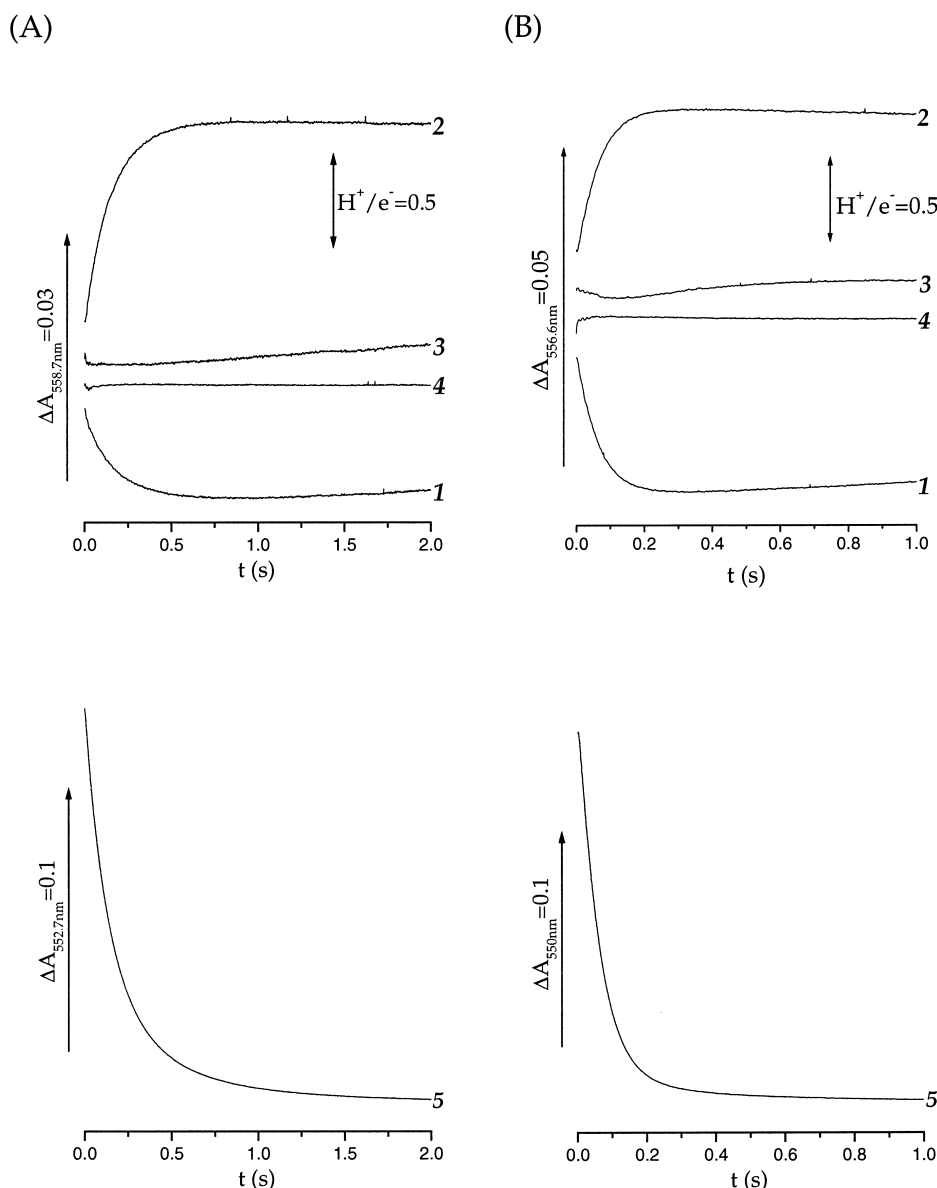


Fig. 4. Proton pumping assay using a stopped-flow spectrophotometer. pH changes were monitored by following the change in absorbance of phenol red at the isosbestic point of the respective cytochrome *c*, oxidation of cytochrome *c* was observed at the wavelength of the α -band maximum. The buffers outside and within the vesicles were as described for Fig. 3, with the external buffer additionally containing 55.5 μ M phenol red (all concentrations after mixing). A: Trace 1: Reaction of 10 μ M reduced cytochrome *c*₅₅₂ with 0.5 μ M *Th. thermophilus* cytochrome *c* oxidase reconstituted into proteoliposomes, followed at 558.7 nm. Trace 2: Experiment as in trace 1, in the presence of 5 μ M CCCP. Trace 3: As in trace 1, but using oxidized cytochrome *c*₅₅₂. Trace 4: As trace 1, without phenol red. Trace 5: As trace 4, absorbance changes monitored at 552.7 nm. B: Trace 1: Reaction of 12.7 μ M reduced horse-heart cytochrome *c* oxidase reconstituted into proteoliposomes, followed at 556.6 nm. Trace 2: As trace 1, in the presence of 5 μ M CCCP. Trace 3: As trace 1, with oxidised cytochrome *c*. Trace 4: As trace 1, without phenol red. Trace 5: As trace 4, absorbance changes monitored at 550.0 nm. Calibration of the signals in A and B was done under the same conditions as for traces A3 and B3, by hydrolyzing known amounts of TAME through mixing with 3.6 μ M trypsin.

been shown to be indispensable for proton pumping in other heme/copper-containing terminal oxidases, there must be mechanistic and/or structural variations to allow the *Th. thermophilus* enzyme to pump protons. This would also be in agreement with the different pump stoichiometries found for the *P. denitrificans* and *Th. thermophilus* cytochrome *c* oxidases. However, further characterization of the *ba*₃ enzyme under various conditions (pH, temperature) and detailed structural information will be necessary to test the validity of this suggestion.

Acknowledgements: We are grateful to Hannelore Müller for excellent technical assistance, to Ron Clarke and Christian Lüpfer for help with the stopped-flow spectrophotometer and to Maarten Ruitenber, Axel Harrenga and Julia Behr for valuable discussions. A grant to G.B. from the Deutsche Forschungsgemeinschaft (Bu 463, 3-2) is gratefully acknowledged.

References

- [1] Ferguson-Miller, S. and Babcock, G.T. (1996) Chem. Rev. 96, 2889–2907.

- [2] Michel, H., Behr, J., Harrenga, A. and Kannt, A. (1998) *Ann. Rev. Biophys. Biomol. Struct.* 27, 329–356.
- [3] Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) *Nature* 376, 660–669.
- [4] Ostermeier, C., Harrenga, A., Ermler, U. and Michel, H. (1997) *Proc. Natl. Acad. Sci. USA* 94, 10547–10553.
- [5] Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) *Science* 272, 1136–1144.
- [6] Hosler, J.P., Shapleigh, J.P., Mitchell, D.M., Kim, Y., Pressler, M.A., Georgiou, C., Babcock, G.T., Alben, J.O., Ferguson-Miller, S. and Gennis, R.B. (1996) *Biochemistry* 35, 10776–10783.
- [7] Pfützner, U., Odenwald, A., Ostermann, T., Weingard, L., Ludwig, B. and Richter, O.-M.H. (1998) *J. Bioenerg. Biomembr.* 30, 89–97.
- [8] Konstantinov, A.A., Siletsky, S., Mitchell, D., Kaulen, A. and Gennis, R.B. (1997) *Proc. Natl. Acad. Sci. USA* 94, 9085–9090.
- [9] Ädelroth, P., Gennis, R.B. and Brzezinski, P. (1998) *Biochemistry* 37, 2470–2476.
- [10] Thomas, J.W., Puustinen, A., Alben, J.O., Gennis, R.B. and Wikström, M. (1993) *Biochemistry* 32, 10923–10928.
- [11] Verkhovskaya, M.L., Garcia-Horsman, A., Puustinen, A., Rigaud, J.-L., Morgan, J.E., Verkhovsky, M.I. and Wikström, M. (1997) *Proc. Natl. Acad. Sci. USA* 94, 10128–10131.
- [12] Ädelroth, P., Svensson-Ek, M., Mitchell, D.M., Gennis, R.B. and Brzezinski, P. (1997) *Biochemistry* 36, 13824–13829.
- [13] Gleißner, M., Kaiser, U., Antonopoulos, E. and Schäfer, G. (1997) *J. Biol. Chem.* 272, 8417–8426.
- [14] Purschke, W.G., Schmidt, C.L., Petersen, A. and Schäfer, G. (1997) *J. Bacteriol.* 179, 1344–1353.
- [15] Keightley, J.A., Zimmerman, B.H., Mather, M.W., Springer, P., Pastuszyn, A., Lawrence, D.M. and Fee, J.A. (1995) *J. Biol. Chem.* 270, 20345–20358.
- [16] Soulimane, T., Gohlke, U., Huber, R. and Buse, G. (1995) *FEBS Lett.* 368, 132–134.
- [17] Soulimane, T., von Walter, M., Hof, P., Than, M.E., Huber, R. and Buse, G. (1997) *Biochem. Biophys. Res. Commun.* 237, 572–576.
- [18] Kleymann, G., Ostermeier, C., Ludwig, B., Skerra, A. and Michel, H. (1995) *Bio/Technology* 13, 155–160.
- [19] Casey, R.P. (1986) *Methods Enzymol.* 126, 13–21.
- [20] Darley-USmar, V.M., Capaldi, R.A., Takamiya, S., Millett, F., Wilson, M.T., Malatesta, F. and Sarti, P. (1987) in: *Mitochondria – A Practical Approach* (Darley-USmar, V.M., Rickwood, D. and Wilson, M.T., Eds.) pp. 143–144, IRL Press, Oxford.
- [21] Bamberg, E., Apell, H.-J., Dencher, N.A., Sperling, W., Stieve, H. and Läger, P. (1979) *Biophys. Struct. Mech.* 5, 277–292.
- [22] Nilsson, T. (1992) *Proc. Natl. Acad. Sci. USA* 89, 6497–6501.
- [23] Geren, L., Hahm, S., Durham, B. and Millett, F. (1991) *Biochemistry* 30, 9450–9457.
- [24] Sarti, P., Jones, M.G., Antonini, G., Malatesta, F., Colosimo, A., Wilson, M.T. and Brunori, M. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4876–4880.
- [25] Sarti, P., Malatesta, F., Antonini, G., Colosimo, A. and Brunori, M. (1985) *Biochim. Biophys. Acta* 809, 39–43.
- [26] Kannt, A., Lancaster, C.R.D. and Michel, H. (1998) *Biophys. J.* 74, 708–721.
- [27] Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M.J., Libeu, C.P., Mizushima, T., Yamaguchi, H., Tomizaki, T. and Tsukihara, T. (1998) *Science* 280, 1723–1730.