Specific cation binding site in mammalian cytochrome oxidase

Anna Kirichenko, Tatiana Vygodina, Hratchik M. Mkrtchyan, Alexander Konstantinov*

A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Building 'A', Moscow 119 899, Russia

Received 13 January 1998; revised version received 23 January 1998

Abstract Calcium ion binds reversibly with cytochrome *c* oxidase from beef heart mitochondria ($K_d \sim 2$ μM) shifting α- and γ-absorption bands of heme *a* to the red. Two sodium ions compete with one Ca^{2+} for the binding site with an average dissociation constant $\sqrt{K_1^{Na} \times K_2^{Na}} \sim 3.6$ mM. The Ca^{2+}-induced spectral shift of heme *a* is specific for mammalian cytochrome *c* oxidase and is not observed in bacterial or yeast aa_3 oxidases although the Ca^{2+}-binding site has been revealed in the bacterial enzyme [Ostermeier, C., Harrenga, A., Ermler, U. and Michel, H. (1997) Proc. Natl. Acad. Sci. USA 94, 10547–10553]. As His-59 and Gln-63 involved in Ca^{2+} binding with Subunit I of *P. denitrificans* oxidase are not conserved in bovine oxidase, these residues have to be substituted by alternative ligands in mammalian enzyme, which is indeed the case as shown by refined structure of bovine heart cytochrome oxidase (S. Yoshikawa, personal communication). We propose that it is interaction of Ca^{2+} with the species-specific ligand(s) in bovine oxidase that accounts for perturbation of heme *a*. The Ca^{2+}/Na^{2+}-binding site may be functionally associated with the exit part of 'pore B' proton channel in subunit I of mammalian cytochrome *c* oxidase.

Key words: Cytochrome *c* oxidase; Proton channel; Calcium binding; Sodium ion

1. Introduction

Cytochrome *c* oxidase (COX) is a terminal oxidase of mitochondria and many bacteria. It catalyses electron transfer from ferrocytochrome *c* to oxygen coupled to transmembrane proton pumping (reviewed [1,2]). In addition to 5 transition metal ions in the redox centres of the enzyme (Cu_A binuclear center, Cu_B, hemes *a* and a_3), the enzyme contains tightly-bound non-redox metal ions – Zn^{2+} and Mg^{2+} (or Mn^{2+}) [3,4].

More than 20 years ago Wikstrom and collaborates discovered that Ca^{2+} shifts absorption spectrum of reduced heme *a* to the red in rat liver and bovine heart COX [5,6]. The finding was confirmed by P. Nicholls [7] and, later on, in this group [8]. Reversal of the spectral perturbation of heme *a* induced by adventitious Ca^{2+} is likely to account for some of the ATP-dependent spectral changes of COX not associated with energization of the membrane [8,9].

Ionic specificity of the calcium effect is peculiar. It was reported initially that none of other metal cations including Eu^{3+}, La^{3+}, Mg^{2+}, Sr^{2+}, Zn^{2+}, Ba^{2+}, Na^+, K^+ or Li^+ could mimick or influence the Ca^{2+}-induced shift [10], but a very similar shift, mutually exclusive with the calcium-dependent one, was induced by protons [5,6,10,11]. It was proposed that Ca^{2+} and H^+ compete for binding with the carboxylic group of propionate substituent in heme *a* [10]. In variance with [10], the Ca^{2+}-induced spectral shift was found later on to be reversed and prevented by sodium ions [12]. Such an ionic specificity (Ca^{2+}/Na^+/H^+) could indicate competition of Ca^{2+}, Na^+ and hydronium cation (H_3O^+) for coordination site (cf. [13]), rather than ionic binding of the metal ions and proton with a single carboxylic group.

Here we show that 1 Ca^{2+} competes with 2 Na^+ for binding with bovine COX and give evidence for competition of Na^+ with protons or H_3O^+. Unexpectedly, we have found that the Ca^{2+}-induced shift is specific for the mammalian enzyme and is not observed with highly homologous oxidases from bacteria or yeast. The latter finding indicates non-equivalence of the Ca^{2+}-binding sites in Subunits I of bacterial and mammalian COX revealed during recent refinement of the crystal structure of the enzyme (Ref. [14], Yoshikawa, personal communication).

2. Materials and methods

$CaCl_2 \times 2H_2O$ was from Sigma. Na_2SO_4 ('special purity' grade) from 'Reachim'. Other chemicals and biochemicals were purchased from Serva, Sigma and Merck. Concentration of Ca^{2+} in stock solutions was checked with murexide titration. COX from beef heart mitochondria was isolated by the method of Fowler et al. [15] with additional purification [16,17]. Yeast COX from *Saccharomyces cerevisiae* was isolated according to [18]. The aa_3-type histidine-tagged COX from *Rhodobacter sphaeroides* was purified on the Ni^{2+}-column (Quiagen) [19] from the membranes kindly provided by D. Mitchell (laboratory of R. Gennis, University of Illinois at Urbana-Champaign) and *Paracoccus denitrificans* COX was a kind gift from Dr. B. Ludwig. Absorption spectra have been recorded in an Aminco-SLM 2000 UV/VIS dual wavelength/double beam spectrophotometer in standard 1-cm rectangular optical cells. COX concentration was determined from the difference (reduced minus oxidized) absorption spectra using extinction coefficient of 27 $mM^{-1}cm^{-1}$ for $\Delta A_{605-630}$ [20].

3. Results

As shown in Fig. 1, Ca^{2+} brings about changes in both visible and Soret bands of the absorption spectrum of bovine heart COX. The lineshape and magnitude of the changes are similar to those reported earlier [6,8,10,12] and indicate a small shift of heme *a* absorption bands to the red as confirmed by spectra simulations (not shown). The shift can be fully reversed by EGTA or other chelators (not shown) and titrates by Ca^{2+} with an apparent K_d that varied in different experiments around 2 μM (1.3–3 μM) (Fig. 2A). This value is significantly less than 20–30 μM as reported earlier [6,12]. The effect of Ca^{2+} on heme *a* is reversed by Na^+ and, in the presence of added Na^+, more Ca^{2+} is required to saturate the spectral shift (Ref. [12], and see below). Presumably, so-

*Corresponding author. Fax: (7) (95) 939 03 38.
E-mail: konst@libro.genebee.msu.su

Abbreviations: COX, cytochrome *c* oxidase

PII S0014-5793(98)00117-3

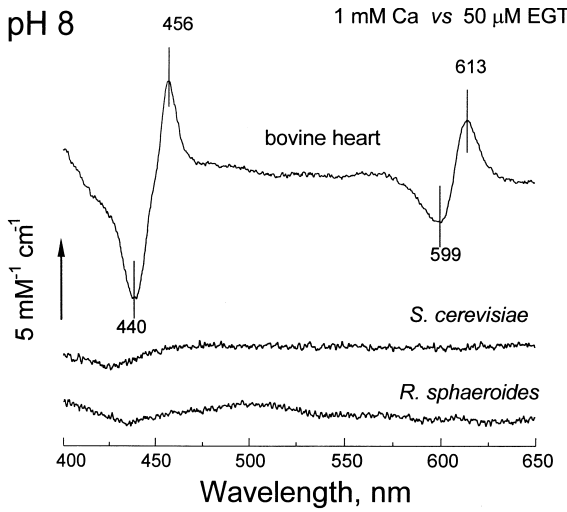

Fig. 1. Effect of Ca^{2+} on the absorption spectrum of cytochrome *c* oxidase from different species. 1.5 μM COX from bovine heart, bacteria or yeast was incubated for 20–30 min in the buffer containing 50 mM Tris-HCl pH 8, 0.045% *n*-dodecyl-β-maltoside and 50 μM EGTA-Tris. Subsequently, 2 mM KCN, 5 mM potassium ascorbate and 100 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine have been added to convert the enzyme to mixed-valence state ($a^{2+}a_3^{3+}$-CN). The resulting mixture was divided between the sample and reference cells and the baseline was recorded. The difference spectra show absorption changes induced by addition of 1 mM Ca^{2+} to the sample.

dium ions were not excluded from reaction mixture in earlier titrations of the Ca^{2+}-induced red shift of COX and this explains the high apparent K_d values obtained.

As shown in Fig. 2B, effective affinity of COX for Ca^{2+} decreases as a regular function of Na^+ concentration. Remarkably, the $K^{eff}_{d(Ca)}/[Na^+]$ plot is not linear but rather follows square dependence indicating competition of 1 Ca^{2+} with 2 Na^+. Non-linear regression analysis of the data assuming 2 independent Na^+-binding sites, each competing with Ca^{2+}

$$K^{Ca}_{eff} = K^{Ca} \times (1 + [Na^+]/K^{Na}_1 + [Na^+]/K^{Na}_2 + [Na^+]^2/(K^{Na}_1 \times K^{Na}_2)) \quad (1)$$

gives the values of K_1 and K_2 for the two sodium-binding sites of 1 mM and 15 mM. However, a reasonably good fit is actually obtained for any pair of K_1,K_2 values within the 1–15 mM range with a mean geometrical $\sqrt{K_1K_2} \sim 3.6$ mM (e.g. dashed line in Fig. 2B). Extrapolation of the dependence in Fig. 2B to zero $[Na^+]$ gives K_d for Ca^{2+} of 2 μM which agrees well with the values directly observed in the titrations without added Na^+ (e.g. Fig. 2A).

Although Na^+ competes with Ca^{2+}, it does not affect itself the spectrum of the Ca^{2+}-free COX at neutral or alkaline pH [12]. However, at acidic pH, Na^+ brings about a *blue* shift of

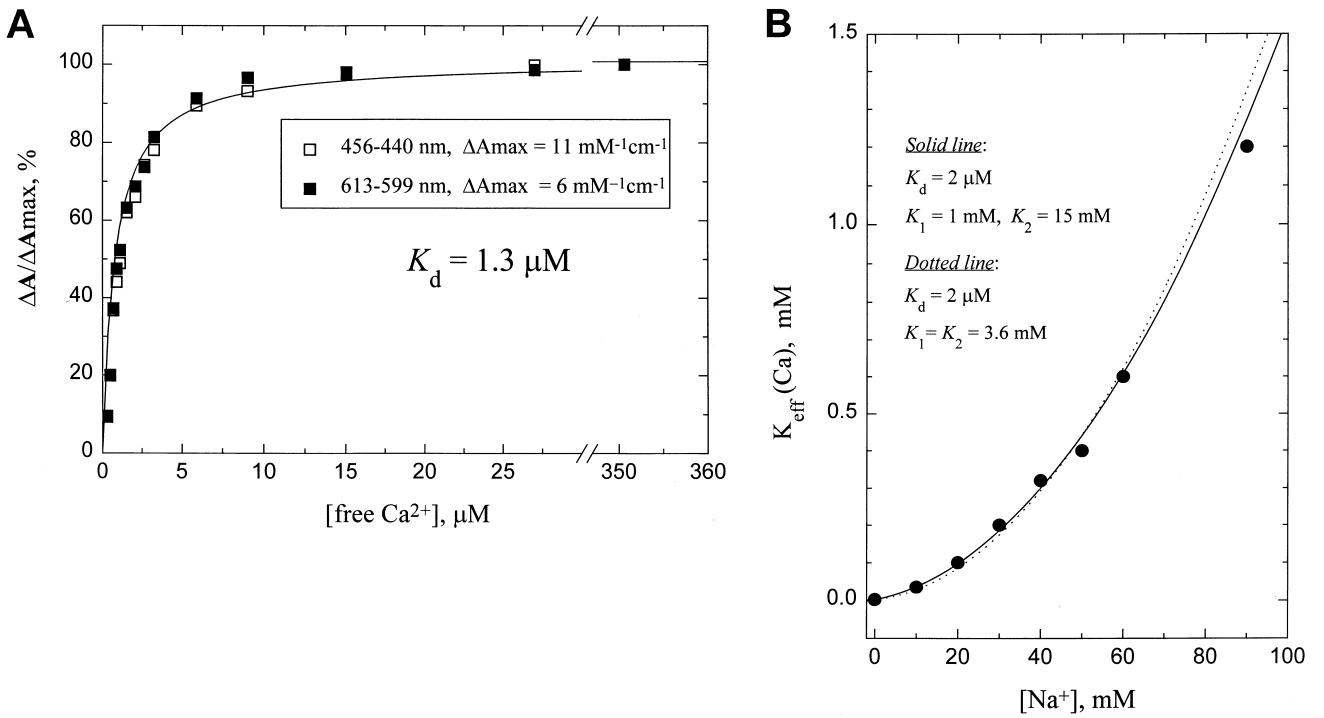

Fig. 2. Competition between Ca^{2+} and Na^+ for binding with COX. A: Titration of the heme a^{2+} spectral shift with Ca^{2+}. Bovine COX (1.5 μM) in 50 mM Tris-MES buffer pH 8 with 0.045% *n*-dodecyl-β-maltoside was reduced by 5 mM potassium ascorbate+0.1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine in the presence of 2 mM KCN. The spectral shift of heme *a* has been titrated by addition of known concentrations of $CaCl_2$ in the presence of 10 mM potassium nitrile triacetate (NTA) as Ca^{2+}-buffer. Concentration of free Ca^{2+} at a given concentration of the added cation has been estimated using K_d(Ca) of 24 μM for NTA at pH 8 (calculated from pH-independent $pK_{Ca} = 6.5$ and $pK_H = 9.8$ [33]). $\Delta A_{peak-to-trough}$ in the difference spectra induced by Ca^{2+} in the visible and Soret band are given normalized to the maximal response observed at saturating concentrations of free Ca^{2+}. The experimental points have been approximated by a hyperbolic curve for a single type of binding site with K_d of 1.3 μM. B: Apparent dissociation constant for Ca^{2+} as a function of Na^+ concentration. Spectral shift of COX was titrated by Ca^{2+} (without NTA buffer at $[Na^+] > 10$ mM) in the presence of different concentrations of added Na_2SO_4. The concentration dependence was linearized to find apparent K_d values for Ca^{2+} and the values found have been plotted as a function of sodium concentration. Assuming a model with 2 independent binding sites for sodium ions (Eq. 1), the points are best fitted with K_1 and K_2 for Na^+ of 1 and 15 mM, respectively, and K_d for Ca^{2+} of 2 μM (solid line), but a good fit is obtained also for $K_1 = K_2 = 3.6$ mM (dotted line).

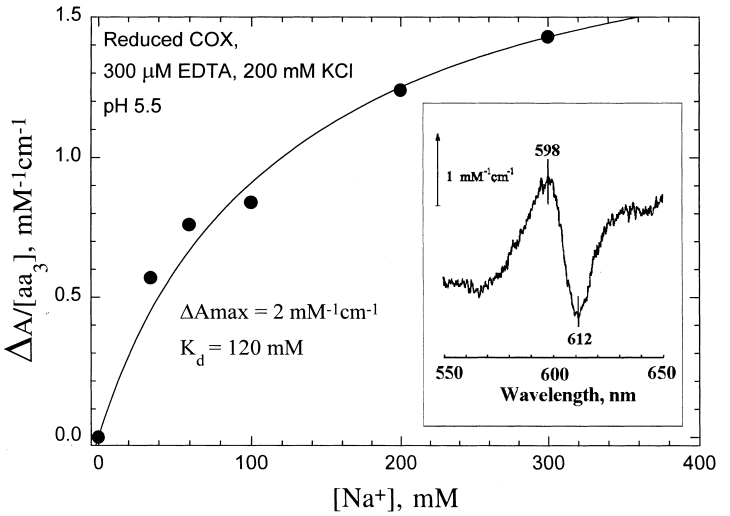

Fig. 3. Reversal of the proton-induced red shift of heme *a* by Na^+. Inset: a blue shift of heme *a* induced by addition of 200 mM NaCl to dithionite-reduced COX (1.1 μM) at acid pH. The buffer contained 25 mM MES+25 mM acetate/KOH pH 5.3, 200 mM KCl and 300 μM potassium EDTA. Main panel: concentration dependence of the Na^+-induced blue shift shown in the inset. Conditions the same as in the inset, except that pH is 5.5.

ferrous heme *a* absorption band which titrates with $[Na^+]_{1/2} \sim 120$ mM at pH 5.5 (Fig. 3). This observation is consistent with the proposal that H^+ (or H_3O^+ [12]) binds with COX at the same site as Ca^{2+} and induces a similar red shift of heme *a* [10]. Accordingly, Na^+ may be expected to compete with both proton and calcium and to reverse the red shift of heme *a* induced by protonation as it reverses the Ca^{2+}-induced effect. According to our observations (experiments in collaboration with Drs. A. Musatov and V. Berka in the Institute of Experimental Physics, Kosice, Slovakia), p*K* of the group in the reduced and mixed-valence COX is ~5.5, that is about 1/2 pH unit more acidic than the values reported by Saari et al. [10]. This explains why our previous attempts to reverse the proton-induced red shift of heme *a* by Na^+ performed at pH 6, in accordance with p*K* reported by the Helsinki group [10], gave negative results [12].

To our surprise, Ca^{2+} in concentrations up to 10 mM does not perturb the spectrum of highly homologous aa_3 oxidases from bacteria (*R. sphaeroides*, Fig. 1; *P. denitrificans*, data not included) and yeast (Fig. 1). Also addition of a large excess of EGTA or Na^+ to COX from *R. sphaeroides, P. denitrificans* or *S. cerevisiae* did not induce a blue shift of heme *a*; this excludes the possibility that the spectral shift is fully developed in the bacterial/yeast enzyme due to adventitious Ca^{2+} prior to addition of the external cation. Apparently, either Ca^{2+} does not bind bacterial/yeast COX or the binding does not affect the spectrum of heme *a*.

4. Discussion

4.1. Relationships between Ca^{2+}, Na^+ and H^+ (or H_3O^+)

Our data confirm competition between Ca^{2+} and Na^+ for binding with COX [12] and indicate that 1 Ca^{2+} competes with 2 Na^+ ions. Evidence for competition between Ca^{2+} and H^+ was given earlier [10]. Finally, a blue shift of heme *a* induced by Na^+ at acid pH (Fig. 3) may point to competition between Na^+ and H^+ (or H_3O^+).The simplest interpretation is that all the 3 cations compete for the same binding site in COX.

4.2. Identity of the Ca^{2+}/Na^+-binding site

The original conclusion that Ca^{2+} and H^+ bind to carboxylic group of the propionate substituent in heme *a* is not corroborated by our data and is difficult to reconcile with the 3D structure of bovine COX [21].

After this work was done, a new metal (supposedly Ca^{2+}) binding site has been revealed in Subunit I of COX from *P. denitrificans* [14] and bovine heart (S. Yoshikawa, personal communication). The site is formed essentially by the residues at the *P*-side end of transmembrane helix I and is much closer to heme *a* than to heme a_3 which accords with the perturbation of the spectrum of heme *a* but not of heme a_3 by calcium. Although Ca^{2+} itself has not been resolved unambiguously in the crystal structure at 2.7 Å resolution, it is most probably bound at this site [14], at least in bovine COX where the locus structure has been refined to 2.3 Å resolution (S. Yoshikawa, personal communication).

It is remarkable that while the new cation binding site is found in both bacterial and bovine COX, the Ca^{2+}-induced spectral shift is observed in the mammalian enzyme only. That the Ca^{2+}-binding site in the 3D structure is totally different from the one responsible for the spectral shift cannot be ruled out formally but is very unlikely. Rather our data indicate that the Ca^{2+}-sites in the bacterial and mammalian enzymes are not identical. Indeed, whereas Glu-56 and Gly-61 proposed to donate 3 of the 5 coordination bonds to Ca^{2+} in *P. denitrificans* [14] are conserved in Subunit I of bovine COX (Glu-40 and Gly-45, respectively), the *Paracoccus* residues His-59 and Gln-63 do not have counterparts in mammalian COX. Accordingly, at least one coordination bond to Ca^{2+} in bovine COX is provided by a residue from the helix XI–XII loop of Subunit I (Yoshikawa, personal communication). Sequence alignment shows that this residue is missing not only in the bacterial, but also in yeast and plant COX. Thus the species specificity of the Ca^{2+}-induced spectral shift matches that of the metal binding site structure and suggests that it is interaction of Ca^{2+} with the non-conserved residue in the XI–XII loop which is a prerequisite for specific perturbation of heme *a* spectrum in mammalian COX.

4.3. Which cation occupies the Ca^{2+}-binding site in COX under physiological conditions?

According to our experience, adventitious Ca^{2+} in solutions kept in glassware can be as high as 5–10 μM, unless special precautions are taken. As Ca^{2+} affinity of COX corresponds to $K_d \sim 2$ μM, the metal binding site in bovine COX is likely to be almost saturated by Ca^{2+} in vitro, if chelators or Na^+ are not present in the buffers. On the other hand, cytoplasmic free Ca^{2+} is typically about 0.1 μM and usually does not rise above 1 μM [22]. This is well below affinity of COX for Ca^{2+} (note that it is cytoplasmic rather than intramitochondrial calcium that interacts with COX as demonstrated by Wikstrom and Saari [6] and confirmed by location of the site in 3D structure) (Ref. [14], S. Yoshikawa, personal communication). Moreover, at cytoplasmic concentration of Na^+ (5–10 mM), effective K_d for Ca^{2+} will rise to 15–35 μM (Ref. [12], Fig. 2B). These considerations question occupation of the cation-binding site by Ca^{2+} in vivo. At the same time, affinity for Na^+ ($K_1 \sim 1$ mM, $K_2 \sim 15$ mM, or $\sqrt{K_1 K_2} \sim 3.6$ mM) suggests at least partial saturation of the 'Ca^{2+}-binding site' in COX with sodium ions in the cell. The situation is complicated by a possibility of energy-linked increase in affinity of the site for cations. There is some evidence for energy-linked protonation of the site [5,6], but energy-dependence of Ca^{2+} or Na^+ binding with COX remain to be studied.

4.4. What is the functional role of the Ca^{2+}/Na^+-binding site?

We are not aware of any salient effect of Ca^{2+} on enzymatic characteristics of COX and functional significance of the new metal-binding site remains to be established. Inspection of the bovine COX structure [21] shows that the site is close to the unique stretch of residues 46–51 (TLLGDD) in the helix I–II-connecting loop of Subunit I in bovine COX. This stretch is not conserved in bacteria and even in yeast or higher plant COX and undergoes a redox-linked structural change (movement of Asp-51) as mentioned by S. Yoshikawa at a number of conference talks. We have also noticed proximity of the cation-binding site/Asp-51 locus to the exit of 'pore B', a putative 'third' proton channel interacting specifically with heme *a* (cf. Fig. 10B in [21]). It is tempting to suggest that the unique $Ca^{2+}/Na^+/H^+$ (H_3O^+)-binding site in bovine COX is linked functionally to the exit proton pathway of pore B. Association of the Ca^{2+} binding site in COX with the exit proton well was considered originally by Wikstrom and collaborates [6,10]. It can be speculated that mammalian COX, a heavy-duty proton pump, has a unique highly optimized exit proton pathway. The original cation-binding site present already in bacterial COX [14] could have been transformed by evolution to acquire a new structure associated with stabilization/regulation of this exit proton pathway in the mammalian COX. It is noteworthy that Na^+ ions have been reported to inhibit proton pumping by COX in mitochondria [23]. Incidentally, since concentration of sodium ions was not specifically controlled in most previous studies of COX, variable stoichiometry of proton pumping by COX as reported by different authors (e.g. [24]) could be partly accounted for by variation in [Na^+] concentrations.

Finally, it is tempting to relate the discussion above with potential inter-replaceability of Na^+ and H^+ in the ion-conducting channels of energy-transducing enzymes (reviewed [25–27]). There is evidence for active translocation of Na^+ directly coupled to operation of terminal respiratory oxidases in several bacteria [28–31]. Perhaps in mitochondrial cytochrome *c* oxidase, 'pore B' could operate not as a strictly H^+-specific proton-hopping pathway (an array of protonatable protein groups) as proposed for 'pores A and C' or D- and K-proton channels, respectively [3,14,21], but rather like a gramicidin-type water-filled channel capable of conducting H^+/H_3O^+ or Na^+ (see [13,32] and discussion therein). Depending on physiological needs, COX could use pore B for Na^+/H^+ antiport (e.g. [31]) or Na^+-pumping (cf. [26,28–30]).

Acknowledgements: We would like to thank Dr. S. Yoshikawa for his generous consent to disclose the 3D structure of the Ca^{2+} binding site in bovine COX in July 1997, long before publication, and Dr. H. Michel for kind communication of the residues coordinating Ca^{2+} in *P. denitrificans* oxidase in October 1997. Expert help of Dr. Dmitry Cherepanov in computer exploring the 3D structure of bovine COX [21] is gratefully acknowledged. We are grateful to Drs. R. Gennis and B. Ludwig for providing bacterial oxidases. This work has been supported in part by the grants from the Russian Fund for Basic Research 97-04-49765 (A.A.K.) and 97-04-49144 (T.V.V. and A.K.).

References

[1] Babcock, G.T. and Wikström, M. (1992) Nature 356, 301–309.
[2] Ferguson-Miller, S. and Babcock, G.T. (1996) Chem. Rev. 7, 2889–2907.
[3] Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Nature 376, 660–669.
[4] Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, T., Yaono, R. and Yoshikawa, S. (1995) Science 269, 1069–1074.
[5] Wikstrom, M.K.F. (1974) Ann. NY Acad. Sci. 227, 146–158.
[6] Wikstrom, M. and Saari, H. (1975) Biochim. Biophys. Acta 408, 170–179.
[7] Nicholls, P. (1975) Biochim. Biophys. Acta 396, 24–35.
[8] Konstantinov, A., Vygodina, T., Popova, E., Berka, V. and Musatov, A. (1989) FEBS Lett. 245, 39–42.
[9] Antonini, G., Malatesta, F., Sarti, P. and Vallone, B. (1988) Biochem. J. 256, 835–840.
[10] Saari, H., Pentilla, T. and Wikstrom, M. (1980) J. Bioenerg. Biomembr. 12, 325–338.
[11] Wikström, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase-A Synthesis, Academic Press, New York.
[12] Mkrtchyan, H., Vygodina, T. and Konstantinov, A.A. (1990) Biochem. Int. 20, 183–190.
[13] Boyer, P.D. (1988) Trends Biochem. Sci. 13, 5–7.
[14] Ostermeier, C., Harrenga, A., Ermler, U. and Michel, H. (1997) Proc. Natl. Acad. Sci. USA 94, 10547–10553.
[15] Fowler, L.R., Richardson, S.H. and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170–173.
[16] MacLennan, D.H. and Tzagoloff, A. (1965) Biochim. Biophys. Acta 96, 166–168.
[17] Carrol, R.C. and Racker, E. (1977) J. Biol. Chem. 252, 6981–6990.
[18] Morin, P.E., Diggs, D. and Freire, E. (1990) Biochemistry 29, 781–788.
[19] Mitchell, D.M. and Gennis, R.B. (1995) FEBS Lett. 368, 148–150.
[20] Nicholls, P., Petersen, L.C., Miller, M. and Hansen, F.B. (1976) Biochim. Biophys. Acta 449, 188–196.
[21] Tsukihara, T., Aoyama, H., Yamashita, E., Takashi, T., Yamaguichi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) Science 272, 1136–1144.
[22] Clapham, D.E. (1995) Cell 80, 259–268.
[23] Lam, E. and Tu, S.-I. (1980) Biochem. Biophys. Res. Commun. 96, 196–202.
[24] Capitanio, N., Capitanio, G., Demarinis, D.A., De Nitto, E., Massari, S. and Papa, S. (1996) Biochemistry 35, 10800–10806.
[25] Skulachev, V.P. (1989) J. Bioenerg. Biomembr. 21, 635–647.
[26] Skulachev, V.P. (1994) Biochim. Biophys. Acta 1187, 216–221.
[27] Dimroth, P. (1997) Biochim. Biophys. Acta 1318, 11–51.

[28] Avetisyan, A.V., Dibrov, P.A., Semeykina, A.L., Skulachev, V.P. and Sokolov, M. (1991) Biochim. Biophys. Acta 1098, 95–104.
[29] Kostyrko, V.A., Semeykina, A.Z., Skulachev, V.P., Smirnova, I.A., Vagina, M.L. and Verkhovskaya, M.L. (1991) Eur. J. Biochem. 198, 537-534.
[30] Park, C., Moon, J.-Y., Cokis, P. and Webster, D. (1996) Biochemistry 35, 11895–11900.
[31] Verkhovskaya, M.L., Verkhovsky, M.I. and Wikstrom, M. (1996) Biochim. Biophys. Acta 1273, 207–216.
[32] Akeson, M. and Deamer, D.W. (1991) Biophys. J. 60, 101–109.
[33] Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1986) Data for Biochemical Research, 3rd edn., Clarendon Press, Oxford.