Cytochrome *c* Oxidase as a Calcium Binding Protein. Studies on the Role of a Conserved Aspartate in Helices XI−XII Cytoplasmic Loop in Cation Binding†

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ABSTRACT: The aa_3-type cytochrome *c* oxidases from mitochondria and bacteria contain a cation-binding site located in subunit I near heme *a*. In the oxidases from *Paracoccus denitrificans* or *Rhodobacter sphaeroides*, the site is occupied by tightly bound calcium, whereas the mitochondrial oxidase binds reversibly calcium or sodium that compete with each other. The functional role of the site has not yet been established. D477A mutation in subunit I of *P. denitrificans* oxidase converts the cation-binding site to a mitochondrial-type form that binds reversibly calcium and sodium ions [Pfitzner, U., Kirichenko, A., et al. (1999) *FEBS Lett. 456*, 365−369]. We have studied reversible cation binding with *P. denitrificans* D477A oxidase and compared it with that in bovine enzyme. In bovine oxidase, one Ca^{2+} competes with two Na^+ for the binding, indicating the presence of two Na^+-binding sites in the enzyme, $Na^+_{(1)}$ and $Na^+_{(2)}$. In contrast, the D477A mutant of COX from *P. denitrificans* reveals competition of Ca^{2+} (K_d = 1 μM) with only one sodium ion (K_d = 4 mM). The second binding site for Na^+ in bovine oxidase is proposed to involve D442, homologous to D477 in *P. denitrificans* oxidase. A putative place for $Na^+_{(2)}$ in subunit I of bovine oxidase has been found with the aid of structure modeling located 7.4 Å from the bound $Na^+_{(1)}$. $Na^+_{(2)}$ interacts with a cluster of residues forming an exit part of the so-called H-proton channel, including D51 and S441.

Cytochrome *c* oxidase (COX)[1] is a terminal enzyme of the respiratory chain of mitochondria and many bacteria. The enzyme oxidizes ferrous cytochrome *c* and reduces molecular oxygen to water (reviewed in refs *1*−*5*). COX contains four metal redox centers that carry out electron transfer in a reaction sequence:

cytochrome *c* → Cu_A → heme *a* → heme a_3/Cu_B → oxygen

The crystal structure of cytochrome oxidases from bovine mitochondria (*6*−*8*) and several bacteria, such as *Paracoccus denitrificans* (*9*, *10*), *Thermus thermophilus* (*11*), and *Rhodobacter sphaeroides* (*12*), has been solved with near-atomic resolution. In addition to iron and copper ions, COX contains a number of metal centers that are not redox active. First, there is a Mg^{2+} or Mn^{2+} ion that holds together subunits I and II and possibly is also involved in an exit pathway for water formed by the enzyme (*13*−*15*). Second, the mitochondrial COX contains Zn^{2+} tightly bound to a small, nuclear-coded subunit Vb located at the inner side of the membrane (*6*, *7*). Third, subunit I of cytochrome oxidase from bovine mitochondria and some bacteria has been shown to contain a cation-binding site located near the cytoplasmic face of the enzyme, close to heme *a* (*8*, *10*, *12*).

In mammalian mitochondria, the cation-binding site reversibly binds calcium or sodium (*16*−*19*) with K_d values for each of the cations not too far from their physiological concentrations in the cytoplasm. In contrast, the bacterial oxidases from *P. denitrificans* (*10*, *20*) and *R. sphaeroides* (*12*, *21*) contain tightly bound calcium that is not removed by excess EGTA. Unfortunately, the crystal structure is currently available only for the Na^+-bound form of bovine oxidase (*8*), and there is no experimentally obtained 3D model for the Ca^{2+}-bound form of the enzyme that could be directly compared with the homologous bacterial structure.

The cation-binding site in the cytochrome oxidases is formed by two domains widely separated within the sequence of subunit I as illustrated for the bacterial and mitochondrial COX by the schemes 1 and 2, respectively. All the ligands of the bound cation, except for one, are located within a short stretch of amino acid residues in a cytoplasmic loop

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[1] Abbreviations: COX, cytochrome *c* oxidase; BAPTA, 1,2-bis-(aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethyleneglycol-bis-(beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $Na^+_{(1)}$ and $Na^+_{(2)}$, sodium ions bound in the Cation Binding Centers 1 and 2 of cytochrome oxidase, respectively; NTA, nitrilotriacetic acid; subscript indexes Rs, Pd and Bt denote amino acid numbering in *R. sphaeroides*, *P. denitrificans*, and bovine heart cytochrome oxidase, respectively.

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connecting transmembrane helices I and II of subunit I (the residues that coordinate the cation are marked in bold):

$$R_{-2}\text{-}X_{-1}\text{-}\mathbf{E_0}\text{-}L_1\text{-}X_2\text{-}\mathbf{X_3}\text{-}P_4\text{-}\mathbf{G_5}\text{-}X_6\text{-}\mathbf{Q_7}\ .....R_{-4}\text{-}R_{-3}\text{-}Y_{-2}\text{-}L_{-1}\text{-}\mathbf{D_0}\text{-}Y_1 \quad (I)$$

$$R_{-2}\text{-}A_{-1}\text{-}\mathbf{E_0}\text{-}L_1\text{-}G_2\text{-}Q_3\text{-}P_4\text{-}\mathbf{G_5}\text{-}T_6\text{-}L_7\ .....R_{-4}\text{-}R_{-3}\text{-}Y_{-2}\text{-}\mathbf{S_{-1}}\text{-}D_0\text{-}Y_1 \quad (II)$$

helices I-II cytoplasmic loop *helices XI-XII cytoplasmic loop*

In view of different numbering of the residues in the homologous sequences, we give relative sequence numbers with the conserved glutamate ($\mathbf{E}_0$) and aspartate ($\mathbf{D}_0$) residues as zero starting points in the two regions.

In the bacterial oxidases, $\mathbf{E_0}$ and $\mathbf{Q_7}$ bind to Ca^{2+} by their side-chain terminal oxygens. In addition, residues $\mathbf{E_0}$, $\mathbf{X_3}$, and $\mathbf{G_5}$ coordinate Ca^{2+} with the carbonyl functions of the polypeptide backbone. $\mathbf{X_3}$ is His59 in *P. denitrificans* and Ala57 in *R. sphaeroides*. One more ligand to bound calcium is provided in *P. denitrificans* COX (*20*), as well as in the enzyme from *R. sphaeroides* (*12*, *21*), by a fixed water molecule that is hydrogen-bonded to a conserved aspartic residue $\mathbf{D_0}$ (D477 in *P. denitrificans*, D485 in *R. sphaeroides*) located in the periplasmic loop between helices XI and XII. This residue is referred below as a "distal aspartate" ligand of the cation. Thus, Ca^{2+} may be thought to connect two domains of subunit I of COX located near the N- and C-termini.

A very similar arrangement of the cation-binding site is observed for bovine COX (Scheme II), except that the structure has been solved in this case for the Na^+-bound form of the enzyme, and accordingly, ligation pattern of this cation differs somewhat from that observed for tightly bound Ca^{2+}. In particular, it is not the conserved aspatate $\mathbf{D_0}$, but an adjacent serine $\mathbf{S_{-1}}$ in the XI−XII cytoplasmic loop that forms a bond with the sodium ion.

Site-specific mutagenesis studies (*20*) showed that replacement of D477 for alanine in subunit I of COX from *P. denitrificans* ($\mathbf{D_0}$ in Scheme I) resulted in a loss of tightly bound calcium by the bacterial oxidase. Concurrently, the enzyme aquired an ability to bind calcium reversibly, like the mammalian COX, with K_d of ca. 10^{-6} M, close to the value determined for bovine heart oxidase (*19*). Binding of calcium with the D477A mutant form of COX induces a red shift of heme *a* absorption spectrum, like in the mammalian oxidase, and the shift is reversed upon addition of excess EGTA. Analogous observations have been made subsequently with the homologous D485A mutant of COX from *R. sphaeroides* (*21*), except that a much tighter binding of calcium ($K_d = 6$ nM) was obtained. Reversible binding of calcium has been reported also for the mutants in the residues E56 and Q63 of *P. denitrificans* oxidase (*22*), that is, $\mathbf{E_0}$ and $\mathbf{Q_7}$ in Scheme I (but cf. ref *20*). The calcium-induced red shift of heme *a* in D477A and E56Q mutant forms of *P. denitrificans* oxidase is reversed by Na^+ ions (*20*, *22*) which are assumed to compete with Ca^{2+} for the binding site by analogy with the mitochondrial oxidase.

Initially, competition of one Ca^{2+} with one Na^+ for the same cation-binding site of cytochrome oxidase was tacitly assumed (*17*, *18*). This point of view agreed with the 3D crystal structures available for several oxidases (*8*, *10*, *12*), in which a single cation-binding center has been resolved, occupied by Na^+ in bovine oxidase and by Ca^{2+} in bacterial oxidases. Below, we refer to this center as Cation Binding Site 1. However, the apparent K_d for calcium binding with the mitochondrial oxidase was found to decrease proportionally to the *square* of sodium concentration, $[Na^+]^2$, rather than to $[Na^+]$ as expected for competition of one Ca^{2+} with one Na^+ (*19*). The parabolic plot $K_d(Ca^{2+})_{obs}$ versus $[Na^+]$ was best-fitted by competition of Ca^{2+} with two sodium ions with the two different $K_d(Na^+)$ values around 1 and 15 mM (*19*). The 1 Ca^{2+}/2 Na^+ stoichiometry looks reasonable from the point of view of maintaining charge balance in the cation-binding site. On the other hand, the finding raised questions as to the nature and location of the second sodium-binding site in the mitochondrial oxidase and has stimulated our interest in further investigations into the characteristics of Ca^{2+} and Na^+ interaction with the cation-binding site(s) in COX.

In this work, we aimed to compare the cation-binding characteristics of the bacterial and mitochondrial oxidases with a view to get an insight into the nature of the second Na^+-binding site in bovine oxidase. Preliminary evidence for the absence of the second sodium-binding site in the *R. sphaeroides* COX mutant lacking the "distal aspartate" D485 was obtained in our recent work (*21*). However, the behavior of this mutant oxidase revealed some deplorable complications, so in the present work, we have turned to the homologous D477A mutant of *P. denitrificans* with a hope to get a simpler experimental model. A limited number of experiments was also made with the Q63A mutant of the enzyme briefly characterized previously (*20*, *22*), to evaluate potential usefulness of this mutant for our studies.

First, Ca^{2+} and Na^+ interaction with the mutant forms of COX from *P. denitrificans* was characterized in some detail. The equilibrium K_d and the *on* and *off* rate constants for Ca^{2+} binding with the D477A mutant of *P. denitrificans* oxidase are found to be not much different from those determined for bovine oxidase, which facilitates comparison between the bacterial and mitochondrial enzymes, whereas such a comparison was complicated by the very tight binding and very slow *off* rate in case of the homologous D485A mutant of *R. sphaeroides* COX (*21*).

As in the case of bovine oxidase, Na^+ itself does not induce the red shift of heme *a* absorption bands in D477A mutant form of *P. denitrificans* oxidase stripped of endogenous calcium, which differs from the behavior of the E56Q mutant in the cation-binding site described in ref *22*, but at high concentrations it brings about a small *blue* shift of heme *a* spectrum. The effect is additive with the Ca^{2+}-induced red shift and cannot be explained by reversal of the red shifts induced by calcium or protons. Like in COX from animal mitochondria, Na^+ reverses the red shift of heme *a* induced in the mutant bacterial oxidase by Ca^{2+} and competes with Ca^{2+} for binding with the oxidase. However, in the D477A mutant of *P. denitrificans* COX, one Ca^{2+} competes with one Na^+, whereas in the bovine COX, competition of one Ca^{2+} with two Na ions is observed. This finding hinted at the possible involvement of the conserved aspartic residue D442 in the helices XI−XII cytoplasmic loop (homologous to D477 in *P. denitrificans* and D485 in *R. sphaeroides*) in the binding of the second Na ion in case of the bovine COX.

Indeed, inspection of the published 3D structures of the oxidized and reduced bovine COX has allowed to suggest a likely binding site for the second sodium ion, $Na^+_{(2)}$. The site involves D442 carboxylate and, notably, a cluster of residues, such as S441, D51 in subunit I, and S205 in subunit

II, that are believed to form an exit part of the so-called H-proton pathway in bovine oxidase (*23*). Structure modeling of the cation-binding site in bovine COX made in this work (and cf. ref *22*) and comparison with the homologous Ca^{2+}-binding site in the bacterial oxidases suggest that upon binding of Ca^{2+} in the Cation Binding Site 1 of bovine COX, D442 can move and form a coordination bond with the bound Ca^{2+} via a fixed water molecule, whereas the "distal" aspartate does not interact with the sodium ion $Na^{+}_{(1)}$ bound in the same site (*8*). Alternative interaction of D442 with Ca^{2+} in Cation Binding Site 1 or $Na^{+}_{(2)}$ in Cation Binding Site 2 can provide a simple explanation for competition between $Na^{+}_{(2)}$ and calcium.

Finally, evidence has been obtained for redox-dependent change of bovine COX affinity for sodium in the second binding site. This effect is likely to explain an apparent ca. 2.5-fold increase in the affinity of COX for Ca^{2+} observed upon reduction of heme *a* (and Cu_A) when experiments are carried out at Na^+ concentrations above ca. 10 mM, but not in the absence of added Na^+.

The potential role of Ca^{2+} and Na^+ in regulation of the H-proton-conducting pathway in the animal mitochondrial oxidase is discussed.

MATERIALS AND METHODS

Chemicals. Calcium chloride dihydrate (min 99.0%) and BAPTA (1,2-bis[aminophenoxy]-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) were from Sigma. EGTA (free acid, ultrapure grade) and NaCl (high-purity grade) were from Amresco (Solon, OH). Dodecylmaltoside was from Anatrace.

Preparations. *P. denitrificans* strains carrying D477A and Q63A substitutions in subunit I of COX were obtained as described earlier (*20*). Cytochrome *c* oxidase was prepared from the membranes of the cells as described in ref *24*. COX from bovine heart mitochondria was purified essentially according to refs *25* and *26*. The preparations were stored in liquid nitrogen. A limited number of control experiments were made with the freshly prepared enzyme to check for possible effects of freezing/thawing and storage of the enzyme in the frozen state.

Measurements. Spectrophotometric measurements were made in an SLM-Aminco 2000 dual-wavelength/split beam spectrophotometer or CaryBio-300 (Varian) split beam spectrophotometer in standard 1 cm rectangular cells. Titrations of cytochrome *c* oxidase absorption shift with calcium were made as earlier (*19*) with the use of calcium buffers such as nitrilotriacetic acid (NTA) or BAPTA and employing Internet-available program "WinMAXC, v. 2.05" written by C. Patton in Stanford University to calculate concentration of free calcium under given conditions, including concentrations of different chelators, added calcium, and other cations, pH, temperature, and ionic strength. The Ca^{2+}-induced shift in the position of the optical absorption bands of heme *a* is rather small (ca. 1 nm in the α-band and even less in the Soret region). Therefore, the amplitudes of the difference absorption spectra (A_{max} minus A_{min}) brought about by Ca^{2+} binding/dissociation were used to quantitate cation binding with the site (cf. refs *17*, *19*, *22*). Effective K_d values for Ca^+ and Na^+ binding with COX were determined from the series of the corresponding difference spectra by a hyperbolic curve-fitting of the titration curves ($\Delta A_{max-min}$ vs [free cation]) with the aid of Microcal Origin 4 or 7Pro software (Microcal Software, Inc.).

Modeling the Cation-Binding Centers in Bovine Cytochrome c Oxidase. Atomic coordinates for both the oxidized and reduced states of bovine cytochrome *c* oxidase (structures 2OCC and 1OCR, respectively, (*8*)) were used as a starting point to model the Cation Binding Sites 1 and 2 in the Ca^{2+}- and Na^+-bound forms of the enzyme. A minimal perturbation approach was used in the modeling, introducing no modification of the main chain and only changing the *chi* dihedral angles of several side chains. The structures for the cation-binding centers obtained in this way are not expected to have perfect coordination geometry. Optimization of the latter would involve, in addition, minor modifications of the main chain with the use of energy minimization methods. However, the energy minimization approach requires defining the exact bonds connecting the metal ions to the protein and to water molecules (considering only the nonbonding interactions would be incorrect). This would inevitably introduce some arbitrary decisions and bias the results, so we preferred to avoid it.

RESULTS

Spectral Shifts of Heme a Induced by Calcium and Sodium in the Mutant Forms of COX. Figure 1 shows difference absorption spectra induced by cations in *P. denitrificans* cytochrome *c* oxidase. In agreement with previous reports (*19*, *20*, *22*), addition of either Ca^{2+} or EGTA to the wild-type oxidase from *P. denitrificans* does not result in spectral changes (trace *a*). In contrast, when Ca^{2+} is added to a mutant oxidase with D477A replacement, a typical red shift of heme *a* absorption band is observed (trace *b*) with a difference spectrum (A_{max} at 613 nm, A_{min} at 599 nm, $\Delta A_{max-min}$ ca. 4 mM^{-1} cm^{-1}) very similar to that described for bovine oxidase.

Sodium ions added to the D477A form of COX pretreated with excess chelator to deplete endogenous calcium do not induce an analogous red shift in contrast to the data reported for the E56Q mutant (*22*). Rather, a small blue shift is observed (trace *d*). At the same time, sodium ions reverse the red shift induced by calcium ions (trace *c*) due to competition between Ca^{2+} and Na^+ for the binding site.

The Q63A mutant of COX from *P. denitrificans* was found earlier to retain bound Ca^{2+} (*20*). At the same time, addition of Ca^{2+} to the Q63A form of oxidase was reported to bring about a red shift of heme *a* (*22*), although the magnitude of the corresponding difference spectrum ($\Delta A_{max-min}$ ca. 1.5 mM^{-1} cm^{-1} in the α-band) was relatively small as compared to the typical effect induced by Ca^{2+} in bovine COX or in the D477A mutant of *P. denitrificans* oxidase ($\Delta A_{max-min}$ = 4−6 mM^{-1} cm^{-1}). In our hands, addition of Ca^{2+} to freshly isolated Q63A COX pretreated with EGTA did not induce spectral shift of heme *a*. Accordingly, the homologous mutant Q61A of *R. sphaeroides* oxidase retains bound calcium and does not reveal spectral shift of heme *a* (*21*). If the sample of Q63A oxidase was kept frozen after isolation, a minor spectral shift of heme *a* induced by Ca^{2+} could be observed (data not shown), consistent with the data in ref *22*; however, this shift was not reversed by excess EGTA. This is in variance with simple behavior of the D477A mutant or bovine oxidase, in which the Ca^{2+}-induced spectral shift is

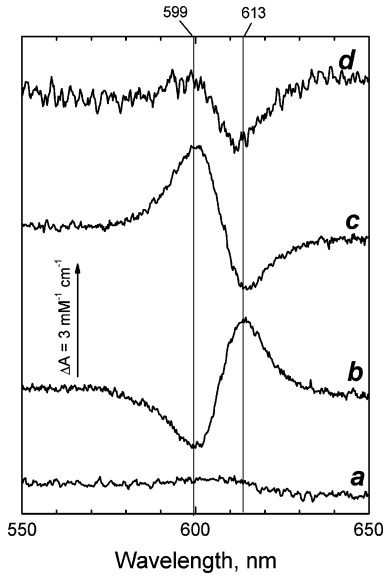

FIGURE 1: Absorption difference spectra induced by binding of calcium and sodium with cytochrome oxidase from *P. denitrificans*. Trace *a*, 1 mM $CaCl_2$ has been added to 1 μM wild-type COX from *P. denitrificans* in a buffer containing 50 mM tris-MES, pH 8.0, with 0.1% dodecyl maltoside and 0.5 mM EGTA-tris. The enzyme was prereduced with 5 mM ascorbate-tris and 100 μM TMPD in the presence of 2 mM KCN. Trace *b*, the same as trace *a*, but with 1 μM D477A mutant form of COX. Trace *c*, reversal of the calcium-induced red shift by Na^+. Initially, 120 μM $CaCl_2$ was added to 1 μM D477A oxidase mutant prereduced with 5 mM ascorbate-tris and 100 μM TMPD in the presence of 2 mM KCN in the buffer containing 50 mM tris-MES, pH 8.0, 0.1% dodecyl maltoside, and 100 μM EGTA-tris. The spectrum was taken as a baseline, and absorption difference induced by subsequent addition of 100 mM NaCl have been recorded. Trace *d*, a blue shift induced by Na^+ in Ca^{2+}-free D477A oxidase. NaCl (500 mM) has been added to 1 μM D477A mutant of COX prereduced with 5 mM ascorbate-tris and 100 μM TMPD in the presence of 2 mM KCN in the buffer containing 50 mM tris-MES, pH 8.0, 0.1% dodecyl maltoside, and an excess of a strong Ca^{2+}-chelator (1 mM BAPTA-tris).

fully reversible by chelators and can be observed with either freshly isolated enzyme or with a sample stored in a deep freezer or liquid nitrogen without any noticeable differences between the data.

Cation-Binding Characteristics. Competition between Calcium and Sodium. In the presence of Na^+, more Ca^{2+} is required to induce the red shift of heme *a* in the D477A mutant of COX (data not shown, see Figure 2C in ref *20*). Titrations of the spectral shift of heme *a* with added Ca^{2+} were carried out at different concentrations of Na^+, and the effective K_d values for Ca^{2+}, determined by a hyperbolic curve-fitting of the titration curves, have been plotted versus sodium concentration (Figure 2A). A straight line is obtained, diagnostic of competition of one Ca^{2+} with one Na^+ for the binding site. The slope of the line in Figure 2 shows that the affinity of the oxidase for Na^+ is ca. 3000-fold lower than for Ca^{2+}, yielding $K_d(Na^+)$ of ca. 4 mM, close to the average geometrical value, $(K_1K_2)^{1/2}$, of the two K_d values determined for binding of the two Na ions with bovine oxidase (*19*).

Similar measurements were performed in parallel experiments with bovine COX (Figure 2B). Calcium affinity was determined for the fully oxidized enzyme and for the partially reduced cyanide complex of COX, in which heme *a* and Cu_A are reduced while the binuclear center is oxidized. There

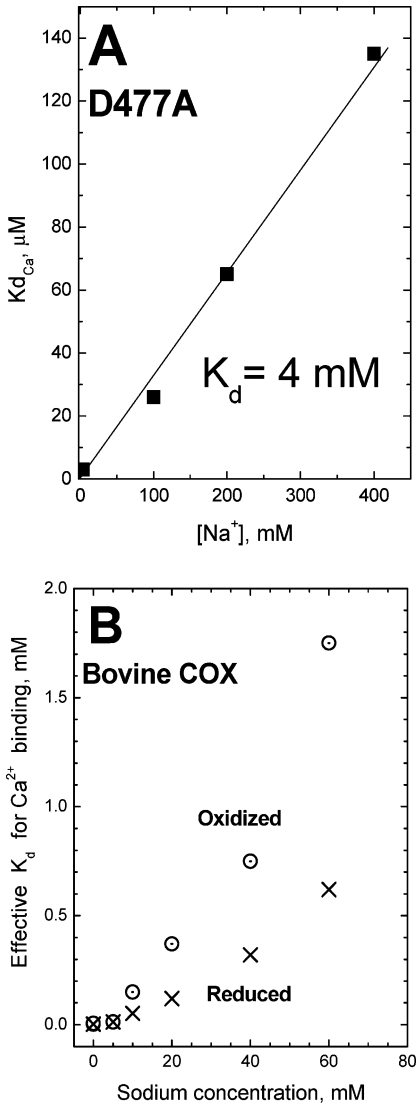

FIGURE 2: Na^+-induced decrease of calcium affinity in cytochrome oxidase from *P. denitrificans* D477A mutant (A) and bovine mitochondria (B). (A) Bacterial oxidase: 1 μM D477A COX from *P. denitrificans* inhibited by 2 mM KCN and reduced by 5 mM ascorbate-tris + 0.1 mM TMPD in 50 mM tris-MES buffer, pH 8.0, containing 0.1% dodecyl maltoside. Ca^{2+}-induced spectral changes were titrated by $CaCl_2$ in the presence of 10 mM NTA-tris calcium buffer at different concentrations of NaCl (in 0−400 mM range). The line corresponds to competition of calcium with one Na^+ binding at a single site with K_d of 4 mM. (B) Mitochondrial oxidase: circles, 2 μM bovine heart COX oxidized by 100 μM ferricyanide in the presence of 5 ug/mL of polylysine (*43*) in a buffer containing 50 mM tris-MES buffer, pH 8.0, 0.1% dodecyl maltoside, and 200 μM EGTA-tris. The spectral changes were titrated with Ca^{2+} until saturation in the presence of different concentrations of NaCl. Crosses, 1 μM bovine COX reduced by 5 mM ascorbate + 0.1 mM TMPD in the presence of 2 mM KCN; other conditions, the same as for the circles. Note exact overlapping of the first two points for the reduced and oxidized COX at 0−5 mM of added Na^+.

are two points worthy of noting. First, the plots of $K_d(Ca^{2+})_{obs}$ versus $[Na^+]$ for the bovine COX in which heme *a* is either reduced or oxidized are no longer linear, but follow parabolic (square) dependence, in agreement with the original observations made with the half-reduced oxidase (*19*); the square dependence indicates competition of one Ca^{2+} with two Na ions. Second, at concentrations of Na^+ above ca. 10 mM, the apparent affinity of calcium for the oxidized COX is

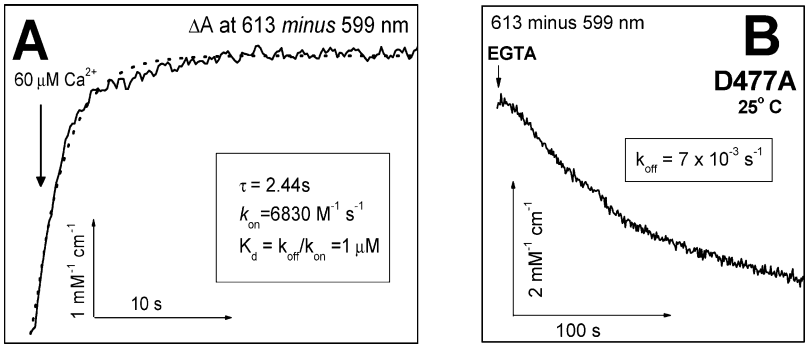

FIGURE 3: Kinetics of calcium binding and dissociation from the cation-binding site in D477A mutant of COX. (A) D477A COX (2 μM) was prereduced with 5 mM ascorbate-tris + 0.1 mM TMPD in the presence of 2 mM KCN in a buffer containing 50 mM tris-MES, pH 8.0, and 0.1% dodecyl maltoside. The enzyme solution was pretreated with 50 μM EGTA to remove endogenous Ca^{2+}, and the reaction was initiated by addition of $CaCl_2$/NTA-tris buffer (final concentration of the free calcium in the cell, 60 μM). Experimental points are fitted with a single exponent (dotted line) with $t_{1/e}$ of 2.44 s. (B) EGTA (200 μM) has been added to 2 μM D477A COX in a buffer containing 50 mM tris-MES, pH 8.0, 0.1% dodecyl maltoside, 2 mM KCN, 5 mM ascorbate-tris, 0.1 mM TMPD, and 100 μM $CaCl_2$.

consistently ~2.5-fold lower (2.6 ± 0.3) than for the enzyme in which heme *a* and Cu_A are reduced. At first glance, this finding might be thought to indicate that reduction of heme *a* (and/or of Cu_A) increases affinity of calcium for the oxidase, for example, for electrostatic reasons. However, titrations in the absence of added sodium or at low sodium concentrations (<5 mM) give the same K_d values for Ca^{2+} binding with the partially reduced or oxidized COX (see the first two points in Figure 2B).

Hence, it is unlikely that the redox state of heme *a* (or Cu_A) directly affects affinity of bovine COX for Ca^{2+}. Rather, there is an indirect effect mediated by redox-dependence of the enzyme affinity for Na^+ ions, which in its turn modulates the apparent K_d for calcium due to the competition of Na^+ with Ca^{2+}. The simplest explanation may be that the oxidized bovine COX has a ca. 2.5-fold higher affinity for Na^+ in the second site (Cation Binding Site 2) with $K_d > 10^{-2}$ M (cf. ref *19*). Accordingly, in the presence of Na^+ at concentrations above the $K_d(Na^+)$ for the second site, the apparent affinity of COX for Ca^{2+} in the oxidized state should decrease relative to the reduced state. The increased affinity of the oxidized bovine COX for Na^+ in Cation Binding Site 2 would be consistent with the structural rearrangements around the residue D51 observed upon the oxidation−reduction of heme *a* (*8*, *27*, *28*) (see Discussion and Figure 6 below).

The kinetics of Ca^{2+} binding with the D477A mutant of COX and Ca^{2+} dissociation from the binding site was studied. The rate of Ca^{2+} binding at low concentrations of Ca^{2+} ($<10^{-4}$ M) allows us to follow the kinetics of the process with hand mixing and converts to the second order *on* rate constant of ca. 7×10^3 M^{-1} s^{-1} (e.g., Figure 3A) that is close to k_{on} determined for *R. sphaeroides* (5×10^3 M^{-1} s^{-1}) (*21*). The off rate for Ca^{2+} dissociation from the site at 25 °C is around 7×10^{-3} s^{-1} (Figure 3B) that is much faster than found for the homologous D485A mutant of COX from *R. sphaeroides* (*21*).

Previous experiments with a D485A mutant of *R. sphaeroides* revealed a ca. 30-fold discrepancy between the values of K_d for Ca^{2+} binding as determined by equilibrium titrations (6 nM) and pre-steady-state kinetics measurements of Ca^{2+} association and dissociation rates ($K_{d(apparent)} = k_{off}/k_{on} =$ 190 nM) (*21*) indicating that Ca^{2+} binding with the D485A form of *R. sphaeroides* COX may involve several elementary steps. In contrast, the K_d determined as k_{off}/k_{on} ratio for the *P. denitrificans* COX with D477A replacement gives a value of ~1 μM, in good agreement with the results of equilibrium titrations (*20*). This observation lends support to the D477A mutant being a "clean" model for comparison with the bovine oxidase, devoid of kinetic complications inherent in the D485A mutant of *R. sphaeroides*.

The ca. 200-fold difference in Ca^{2+} affinity between the D477A mutant oxidase from *P. denitrificans* (ca. 1 μM) and the D485A form of COX from *R. sphaeroides* (ca. 6 nM (*21*)) is per se surprising. It is however difficult to interpret this difference meaningfully in the absence of high-resolution crystal structure for the mutants. Besides, detailed pre-steady-state kinetics studies of Ca^{2+} binding/dissociation with/from the cation-binding site in the D477A mutant of *P. denitrificans* COX, matching those performed with the D485A mutant of *R. sphaeroides* oxidase (*21*), are required for solid comparison between the two bacterial enzymes.

The rate of Ca^{2+} dissociation from the binding site in the D477A mutant of *P. denitrificans* COX is highly temperature-dependent, as found earlier for the D485A mutant oxidase from *R. sphaeroides* (*21*). Activation energy determined from the van't Hoff plot in the range 10−35 °C (Figure 4) is about 23 kcal/mol. The high activation energy for Ca^{2+} dissociation from the binding site in the mutant bacterial oxidases may suggest protein rearrangement (e.g., crevice opening) required for exchange of the bound Ca^{2+} with the aqueous phase.

Na^+/H^+ Relationships. As shown in Figure 1 (trace *d*), Na^+, added to the D477A mutant form of COX preincubated at pH 8 with excess BAPTA (or EGTA, not shown) to remove endogenous Ca^{2+}, brings about a *blue* shift of absorption spectrum of heme *a*. A similar blue shift was observed in the homologous D485A mutant of COX from *R. sphaeroides* (Kirichenko, unpublished observations; (*21*)). At the same time, spectral characteristics of bovine COX are not affected by Na^+ at pH > 6 (*18*), although a blue shift can be induced at more acidic pH (*19*). Three possible explanations of the effect may be considered: (*i*) Na^+ reverses the red-shift induced by calcium ion (*18*); (*ii*) Na^+ reverses the red-shift induced by protons (*17*); and (*iii*) Na^+ itself affects the spectrum of heme *a*.

Possibility (*i*) is unlikely, since the effect of Na^+ is observed in the presence of excess BAPTA or EGTA that

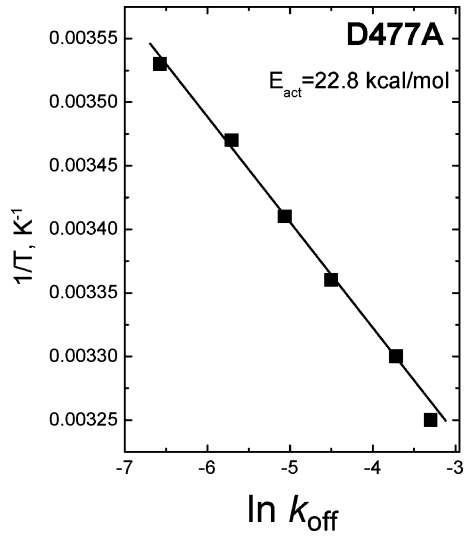

FIGURE 4: Temperature dependence of the rate of calcium dissociation from D477A mutant of *P. denitrificans* COX. The kinetics of Ca^{2+} dissociation from COX was measured in the temperature range 10−35 °C, and first-order rate constants were determined by single-exponential fitting of the curves. The data obtained are presented as the van't Hoff plot. Basic conditions, as in Figure 3B.

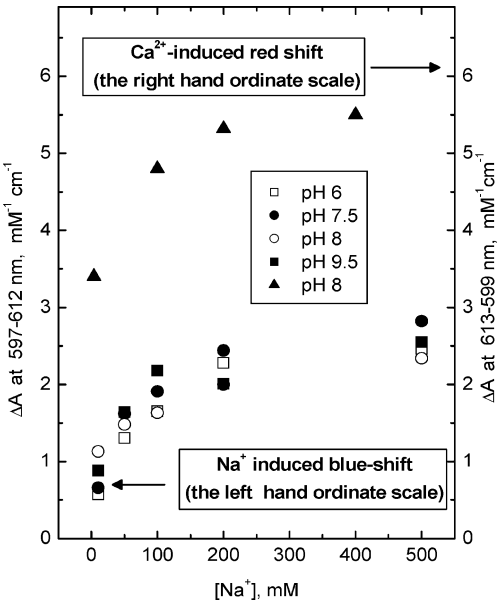

FIGURE 5: pH-independence of the Na^+-induced blue shift in D477A mutant oxidase, and reversal of the Na^+-induced blue shift by calcium. Circles and squares, an amplitude of the Na^+-induced blue shift of heme *a* at different pH values (the left-hand scale ordinate). Basic conditions, as in Figure 1, trace *d*. D477A oxidase (1 μM) was reduced with 5 mM ascorbate-tris + 0.1 mM TMPD in the presence of 2 mM KCN. Titrations of the spectral changes induced by Na^+ were performed in 50 mM MES-tris (pH 6.0, 8.0, and 9.5) or 50 mM MOPS-tris (pH 7) buffers, containing also 0.1% laurylmaltoside, and supplemented with 1 mM BAPTA-tris to remove free Ca^{2+}. Triangles, the maximal amplitude of the Ca^{2+}-induced red shift at different concentrations of Na^+ present initially in the buffer (the right-hand ordinate scale). Conditions, as in Figure 2A.

should have extracted any reversibly bound calcium from the enzyme. To explore the possibility (*ii*), we have studied pH-dependence of the Na^+-induced effect. As shown in Figure 5, the sodium-induced blue shift of heme *a* in D477A COX from *P. denitrificans* grows with increased Na^+ concentration reaching a saturating value (ca. 2.5 mM^{-1} cm^{-1}) at about 500 mM of the cation. Neither the amplitude nor the midpoint of the Na^+ concentration-dependence (ca. 0.1 M) depend on pH significantly. These data are hardly compatible with the reversal of the proton-induced red shift by Na^+ ions. Therefore, we conclude that, in the COX mutants in the "distal aspartate" (**D_0**, Scheme I), Na^+ ions are themselves able to induce a *blue* shift of heme *a*. The blue shift appears to be a novel effect induced by sodium ions binding at a very low-affinity site with apparent K_d around 0.1 M, fully different from the above-described site with $K_d \sim 4$ mM responsible for competition of Na^+ with Ca^{2+}.

As shown in Figure 5, the maximal amplitude of the Ca^{2+}-induced bathochromic spectral shift of heme *a* grows 1.7-fold with increased concentration of Na^+ reaching a saturating value of ca. 5.7 mM^{-1} cm^{-1} in the α-band at [Na^+] above 300 mM (triangles). The sodium-dependent increment in the size of the Ca^{2+}-induced response of ca. 2.5 mM^{-1} cm^{-1} matches the magnitude of the blue shift induced by Na^+, and the two effects show very similar dependencies on Na^+ concentration. It is likely that Ca^{2+} reverses the blue shift induced by high concentrations of Na^+ ions and this reversal adds to the red shift induced by calcium ions alone, that is, in the absence of sodium.

Finally, it is noted that our experiments with the D477A or Q63A mutants of *P. denitrificans* COX have not revealed any indications to a *red* shift of heme *a* induced by Na^+ ions. So the latter may be specific for the E56Q mutant of the oxidase (*22*).

Modeling the Ca^{2+}- and Na^+-Binding Sites in Bovine Cytochrome c Oxidase. As noted above, the crystal structures of cytochrome *c* oxidases of A1-type (see ref *29* for classification) reveal only one cation-binding center, denoted here as Cation Binding Site 1, that contains tightly bound Ca^{2+}, in the oxidases from *P. denitrificans* and *R. sphaeroides* (*10*, *12*), and Na^+, in case of the bovine oxidase (*8*). On the other hand, competition of one Ca^{2+} with two Na^+ for binding with bovine COX ((*19*), this work) pointed to the potential presence of two Na^+-binding sites in the enzyme.

Accordingly, the first objective of our modeling studies was to inspect carefully the published 3D structure of the bovine oxidase and search for a likely binding site for the second sodium ion, referred below as Cation Binding Site 2. Second, we aimed to explore possible alterations in the structure of Cation Binding Site 1 that might be induced by displacement of $Na^+_{(1)}$ in this site by Ca^{2+}. The following intuitive criteria were kept in mind during the search for the $Na^+_{(2)}$ site in bovine COX. First, there should be a cavity fitting the ionic radius of desolvated Na^+ and providing suitable ligands, presumably oxygen atoms, for Na^+ coordination sphere. Second, the site was expected to carry a negative charge and to be located close enough to the Cation Binding Center 1, so that it could provide the second negative charge, in addition to the one donated by $E40_{Bt}$, required for neutralization of bound Ca^{2+}. Third, the model would explain how $Na^+_{(2)}$ coordination at the Cation Binding Site 2 antagonizes binding of Ca^{2+} at the Cation Binding Site 1. Involvement of the "distal aspartate" D442 in the $Na^+_{(2)}$ site formation was tentatively assumed as a starting point.

To model the binding site for $Na^+_{(2)}$ with the aid of the minimal perturbation approach (see Materials and Methods),

coordination sphere of the first sodium ion, $Na^{+}_{(1)}$, was not changed at all, and the second sodium ion was inserted at a distance of 7−8 Å from the first one. To fit this new binding site in the reduced enzyme (Figure 6A), the side chain of Asp442A was rotated by about 180° in *chi*$_1$ and the side chain of Asp51A was moved toward $Na^{+}_{(2)}$ rotating it by ∼30° both in *chi*$_1$ and *chi*$_2$ (the indexes A or B after the residue number denote the polypeptide chain of COX in the database, i.e., subunits I or II, respectively). In addition, the side chain of Ser 205B was adjusted (90° in *chi*$_1$) to allow for a coordination bond with $Na^{+}_{(2)}$, and the side chain of Phe206B was moved away (rotated by about 180° in *chi*$_1$), as it was clashing with the new modeled site. For modeling the second sodium-binding center in the oxidized COX (Figure 6B) that shows substantial redox-dependent structural changes in this region (*8*, *28*), the side chains of both Asp50A and Asp51A were rotated to coordinate the cation (ca. 180° in *chi*$_1$ for Asp 50A; ca. 30° in *chi*$_1$ and 90° in *chi*$_2$ for Asp 51A).

To model the structure of the Ca^{2+}-bound form of the bovine oxidase (Figure 6C,D) we placed Ca^{2+} in Cation Binding Site 1 in the position of the original sodium ion (*8*), just slightly displaced. First, it was noticed that the side chain of Gln43A can provide an additional coordination bond to the cation if rotated by about 180° in *chi*$_1$ toward the calcium ion. Since Tyr104D was bumping with the moved Gln43A, we also changed the dihedral angle of the former side chain (ca. 45° in *chi*$_2$). Second, the side chain of Asp442A, if adjusted by approximately 45° in *chi*$_1$, can bind to Ca^{2+} via an intercalated water molecule, as found for *P. denitrificans* (*20*) or *R. sphaeroides* oxidases (*12*, *21*). Hence, rotation of the D442 side chain may provide for alternating interaction of its carboxylate with either $Na^{+}_{(2)}$ or Ca^{2+} in the disodium- or calcium-bound forms of COX, respectively.

All the changes made to the parent structures in the modeling can be obtained upon request from C.M.S. (e-mail: claudio@itqb.pt).

DISCUSSION

The Mechanism of the Spectral Shift of Heme a Induction by Calcium. The physical origin of the red shift of the absorption spectrum of heme *a* induced by Ca^{2+} in cytochrome *c* oxidase from bovine heart or mutant enzymes from *P. denitrificans* and *R. sphaeroides* has not been established yet. On the basis of the crystal structure of the enzymes, two possibilities were proposed in refs *22* and *30*. First, the effect of Ca^{2+} could be mediated through a highly conserved arginine residue adjacent to the Ca^{2+}-binding loop (R_{-2} in the helices I−II loop in Scheme I, $R38_{Bt}$, $R54_{Pd}$, $R52_{Rs}$) that forms a hydrogen bond to formyl group of heme *a*. Alternatively, the shift could be transmitted through one of the two conserved arginine residues in the helices XI−XII cytoplasmic loop ($R439_{Bt}$, $R474_{Pd}$, $R482_{Rs}$) interacting with propionate group of heme *a* (*30*). This arginine (R_{-3} in Scheme I) is close to the distal aspartate ligand of calcium (**D**$_0$, D477 in *P. denitrificans*) located in this loop.

Our data may favor the first of these alternatives. Indeed, since calcium is able to induce a full-size red shift of heme *a* in cytochrome oxidase mutants devoid of the distal aspartate ligand such as D477A in *P.* denitrificans (this work, (*20*)) or D485A in *R. sphaeroides* ((*21*)), interaction of the cation with the helices XI−XII loop appears to be not essential for induction of the spectral shift. Accordingly, transmission of electronic perturbation through the highly conserved arginine R_{-3} in the helices I−II loop (Scheme I) and its hydrogen bond with the formyl group of heme *a* is favored. A small effect of Ca^{2+} on the stretching frequency of a hydrogen bond of heme *a* formyl group was indeed noticed for the D485A mutant COX from *R.* sphaeroides (*21*).

Competition between Ca^{2+} and Protons. It is believed that calcium ions and protons (or hydronium cations (*18*)) compete for binding with the same cation site in cytochrome oxidase and, accordingly, induce the same red shift of heme *a* upon the binding (*17*−*19*, *22*). In the original model, Ca^{2+} and H^{+} ions were concluded to bind to propionate substituent in heme *a* (*17*). As this hypothesis was not verified by 3D structure of the enzyme, it is worthwhile to consider other possibilities. The two protonatable groups, closest to the cation in the bovine oxidase, are E40 and D442 (*8*) (or the homologous E56 and D477 residues in *P. denitrificans* enzyme (*10*)). Protonation of either of these groups could be responsible for the competition of Ca^{2+} with protons and for the proton-induced red shift of heme *a*. Since the red shift of heme *a* can be observed in the mutants lacking the distal aspartate ($D477A_{Pd}$ or $D485A_{Rs}$), the role of this group protonation is questioned. Protonation of E40 in the main cation-binding loop (or, coordination of H_3O^{+} in the Cation Binding Site 1 (*18*)) is more likely to be responsible for the H^{+}-induced red shift of heme *a* and for competition between H^{+} or/and H_3O^{+} and Ca^{2+} for the binding site.

Competition between Calcium and Sodium. Is D442 Involved in the Second Na^{+}-Binding Site in Bovine Oxidase? When sodium ions were found first to reverse the spectral shift of heme *a* induced by Ca^{2+} in the mitochondrial cytochrome *c* oxidase, it was assumed that Na^{+} binds at the same coordination site as calcium but, in contrast to calcium, does not induce itself the spectral shift of heme *a* (*18*). Accordingly, the binding site for sodium ion resolved in the 3D structure of bovine COX ($Na_{(1)}$ in Figure 6) can accommodate bound Ca^{2+} equally well (*8*) and corresponds to the Ca^{2+}-binding site in bacterial cytochrome oxidases (*10*, *20*, *21*)). However, after Ca^{2+} was found to compete with two sodium ions in bovine oxidase ((*19*), this work), a question emerged as to where is the binding site for the second sodium ion, $Na^{+}_{(2)}$.

Experiments with the bacterial mutant oxidases gave some hints about the answer. In the D477A mutant of *P. denitrificans*, Na^{+} competes with calcium as it does in bovine enzyme, but the plot of $K_d(Ca^{2+})$ versus $[Na^{+}]$ follows a straight line (Figure 2A) showing that, in this case, one Ca^{2+} competes with only one Na^{+}. Provisional indications to the 1:1 competition between Ca^{2+} and Na^{+} was obtained earlier with the D485A mutant COX from *R. sphaeroides* (*21*). As the replacement of the distal aspartate **D**$_0$ (Scheme I) in the mutant oxidases from *P. denitrificans* (this work) and, possibly, *R. sphaeroides* (*21*) concurred with the absence of the second Na^{+}-binding site in the mutant enzymes, it was tempting to suggest that the conserved aspartate in the helices XI−XII cytoplasmic loop ($D477_{Pd}$, $D485_{Rs}$, $D442_{Bt}$) is involved in the binding of $Na^{+}_{(2)}$, at least in the bovine oxidase. It is noted that, despite the similar structures of the mitochondrial and bacterial enzymes, there is no experimental

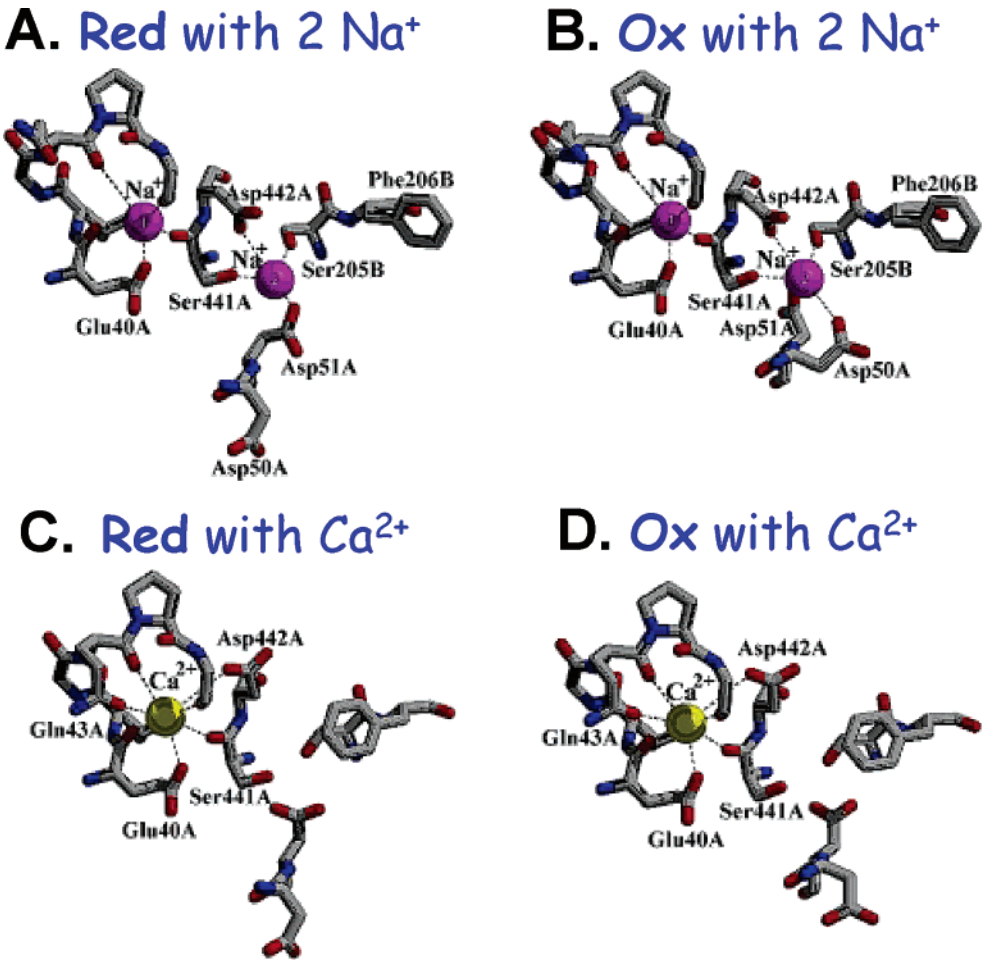

FIGURE 6: A proposed structure of the cation-binding site(s) in bovine cytochrome oxidase at different redox states with one calcium or two sodium ions bound. See the text for details.

evidence yet for the presence of the second cation-binding site in the wild-type bacterial oxidases from *P. denitrificans* or *R. sphaeroides*. Hence, the hypothesis on a specific role of D442 in binding of $Na^+_{(2)}$ with bovine oxidase, as inferred from the experiments with the mutant bacterial oxidases, was strictly speaking just a conditional speculation. But then, it gave a useful impact on the subsequent modeling studies.

Modeling the Binding Site for the Second Na^+ in Bovine Oxidase. Careful inspection of the crystal structure of bovine oxidase allows us to discern a likely place for the second sodium ion-binding site that involves D442 (Figure 6A,B). As described in Results, inclusion of Na^+ at this site requires but minimal perturbation of the atomic coordinates.

In the reduced COX, $Na^+_{(2)}$ is within a coordination bond distance from five oxygen atoms belonging to Ser441, Asp442, and Glu51 in subunit I and Ser205 in subunit II. Water molecules may easily occupy additional coordination positions. The $Na^+_{(1)}$−$Na^+_{(2)}$ distance is 7.4 Å. In the oxidized COX that reveals an altered conformation around the cation binding site (*8, 28*), $Na^+_{(2)}$ may be also within coordination distance from the side-chain oxygens of Asp50 in subunit I. This additional interaction would favor increased affinity of COX for $Na^+_{(2)}$ in the oxidized state, as observed experimentally (see Figure 2B and relevant text in Results).

Asp442 has not been included in the metal cation coordination sphere in the Na^+-bound form of bovine oxidase (*8*). $Na^+_{(1)}$ is coordinated instead by a main-chain carbonyl function of adjacent Ser441 (Scheme II), whereas Asp442 may form a salt bridge with Arg154 of subunit II. However, it is reasonable to presume that, in the Ca^{2+}-bound form of bovine COX, for which the 3D structure has not been reported yet, interaction with ionized Asp442 is required to neutralize the extra positive charge of the bound calcium dication relative to the case of bound Na^+. The interaction is rendered possible by rotation of Asp442 by ca. 45° in chi_1 after which it can form a bond with Ca^{2+}. The bonding is presumed to occur via a fixed water molecule (not depicted in Figure 6) intercalated between the carboxylic group of Asp442 and Ca^{2+}, as shown for the Ca^{2+}-bound forms of the oxidases from *P. denitrificans* and *R. sphaeroides* (*12, 20, 21*), rather than through direct binding of the aspartate carboxylic function with the cation as proposed in ref *22*. Coordination of $Na^+_{(2)}$ by Asp442 should divert the latter from interaction with Ca^{2+} in the Cation Binding Site 1, which provides a simple explanation for the competition of Ca^{2+} with $Na^+_{(2)}$ for binding with the oxidase.

The modeled structure of bovine oxidase, with Ca^{2+} bound in the Cation Binding Site 1 instead of Na^+, is shown in Figure 6C,D. The major predictable change induced in the structure (*8*) by replacement of $Na^+_{(1)}$ by Ca^{2+} involves, besides the above-discussed Asp442 movement, rotation of the side chain of Gln43 enabling it to coordinate calcium by both the side-chain oxygen, as well as by the main-chain carbonyl function (position of the latter remaining unchanged

in the model). This structural adjustment differs from the proposal of Riistama et al. (*22*), who considered only the main-chain oxygen of Gln43 as the calcium ligand and suggested the phenyl function of Tyr443 to be an additional coordinating group. While both alternatives are worthy of consideration, the change in a position of Tyr443 induced by replacement of Na^+ by Ca^{2+} in the Cation Binding Site 1 is less likely, in our opinion, since Tyr443 is much more buried than Gln43, which may be expected to result in lower conformation mobility of the former. Direct ligation of calcium by carboxyl oxygens of $Asp442_{Bt}$ or $Asp447_{Pd}$, with an oxygen−Ca^{2+} distance of 2.34 Å as proposed in ref *22*, is not likely. Refinement of the cation-binding site structure in *P. denitrificans* revealed a fixed water intercalated between the distal aspartate and calcium (*20*) so that the Asp477 carboxyl oxygen−cation distance is 4.6 Å, rather than 2.34 Å as proposed in ref *22*. The ca. 2 Å difference in the distance between the aspartate carboxylate and calcium ion may have entailed significant distortions in the structure of the Ca^{2+}-loaded Cation Binding Site 1 as modeled in ref *22*.

An obvious question is—why the second sodium ion, $Na^+_{(2)}$, revealed in mitochondrial oxidase through its competition with Ca^{2+} ((*19*), this work) and tentatively placed within the 3D structure of COX in this work, has not been identified in the crystal structure of the bovine oxidase (*8*, *28*)?

Two reasonable explanations may be concerned: (1) low concentrations of sodium and (2) acidic pH employed in the crystallographic studies (*8*, *28*). Inspection of the refined structure of the exit part of the H-channel, as presented by Yoshikawa and collaborators at the 12th EBEC Meeting in Arcachon and described in refs *23* and *28*, show that the crystals of the reduced oxidase have been obtained by washing the oxidized enzyme with a buffer containing only 10 mM sodium (5 mM sodium dithionite) which is below affinity of the oxidase for $Na^+_{(2)}$ (K_d ∼15 mM at pH 8, (*19*)). Moreover, pH of the washing buffer in the work of the Japanese group was 5.8, whereas below pH 6, Na^+ begins to compete with protons or hydronium cations for binding with the bovine COX (*17*−*19*).

It is noteworthy that in the refined structure of the H-channel domain in the reduced COX (*23*, *28*), there is a fixed water molecule located close to the site where we place the $Na^+_{(2)}$ cation and coordinated by a similar set of residues (D51, S441 in subunit I and S205 of subunit II). This water observed in acidic crystals might actually be a hydronium cation exchangeable with Na^+ at neutral pH in accordance with a proposed competition between sodium and hydronium cations for the site (*18*).

Physiological Significance of the Second Na^+-Binding Site in Mammalian Oxidase. The proposed binding site for the second Na^+ in subunit I of bovine oxidase locates right inside a cluster of residues whose critical role in the mitochondrial COX catalytic mechanism and/or regulation has been discussed actively in recent years (*8*, *23*, *27*, *28*, *31*, *32*). In particular, $Na^+_{(2)}$ interacts with the side chains of Ser441 and Asp51, the two specific residues absent in the bacterial oxidases or in mitochondrial COX from fungi or higher plants. According to Kadenbach and co-workers (*31*), these two residues might have evolved in animals phylogenetically in a concerted way, supposedly in parallel with phylogenetic appearance of hormonal regulation. Interestingly, S441 and D51 form an exit part of a putative transmembrane proton-conducting pathway denoted as "H-proton channel" (*8*, *10*, *23*, *28*, *33*, *34*). The functional role of this pathway has not been clarified yet. According to a model of the Japanese group (*8*, *23*, 27, 28), the H-channel is involved in transmembrane proton pumping coupled to oxidoreduction of heme *a*. The proton pumping mechanism of bovine COX linked to heme *a* oxidoreduction was proposed originally in refs *35* and *36* and has been reconsidered recently by several laboratories (*28*, *37*−*40*).

A fully different role of the H-channel was proposed by the Moscow group and has been discussed in ref *21*. According to the hypothesis, the H-channel may be a genuine transmembrane proton conducting pathway, but it is involved in proton conduction *down* the protonmotive force, that is, in *dissipation* of membrane potential, rather than in pumping the protons out. Presumably, the channel is normally closed and opens when dictated by physiological needs of the cell, serving as a kind of a safety valve to prevent mitochondrial membrane potential going above the optimal level. As widely discussed in the literature, the so-called "mild uncoupling" of mitochondria is likely to turn on at high protonmotive force values to prevent further increase of membrane potential that can lead to inhibition of respiration and oxygen radical formation (see refs *4*, *31*, *41*) and references therein). The mechanism of "mild uncoupling", however, remained obscure. The H-channel, unless shown eventually to be involved indeed in proton pumping, could be an example of a tightly regulated uncoupling module built into the animal COX and involved in a control over mitochondrial membrane potential, respiration rate, and reactive oxygen species production. Modulation of these processes by cytoplasmic Na^+ and Ca^{2+} through their competitive interaction with the H-channel would be of obvious physiological significance and interest.

Whatever the function of the H-channel, two modes of its regulation by the cations can be envisaged. First, there can be direct effects of Ca^{2+} and Na^+ on the H proton-conducting pathway induced by their binding with the exit part of the channel. Second, it is intriguing that the residue S441, directly interacting with the cations bound in the Cation Binding Sites 1 and 2, is located within a canonical sequence $RRYS_{442}$, a unique one in subunit I of COX, recognized by cAMP-dependent serine/threonine protein kinases (*42*); thus, S441 might serve as a phosphorylation target. It has been proposed by Kadenbach and co-workers (*31*) that phosphorylation/dephosphorylation of S441 switches mitochondrial COX between the nonpumping and pumping states, respectively and that these states differ also in reactivity toward hormones and in the enzymatic activity. Obviously, binding of the cations with S441 should greatly affect reactivity of the residue toward protein kinases, and this might be a second—more sophisticated and more potent—way to control the H-channel.

Finally, although the functional role of the H-channel remains to be established yet, it is hardly possible to believe that such an elaborate intraprotein structure is devoid of important functions. More detailed speculations on the potential effects of Ca^{2+} and Na^+ on the operation of the H-channel would await discovery of the functional role of this proton conducting pathway.

CONCLUSIONS

(1) Ca^{2+} ions induce fully developed red shift of heme *a* absorption spectrum in the mutant of *P. denitrificans* cytochrome oxidase missing residue D477 in subunit I; this residue mediates Ca^{2+} interaction with the cytoplasmic loop connecting transmembrane helices XI and XII in the polypeptide. Accordingly, the calcium-induced spectral shift is unlikely to be transmitted to heme *a* via the conserved residue R474 located close to D477 and interacting with the propionate substituent in heme *a*. The effect may be transmitted via an alternative pathway involving conserved arginine 54 hydrogen-bonded to the formyl group of heme *a* (*22*, *30*).

(2) At high concentrations ($K_d \sim 0.1$ M), Na^+ induces a *blue* shift of heme *a* in calcium-free D477A COX from *P. denitrificans* that cannot be assigned to reversal of a red shift induced by calcium ions or protons.

(3) Na^+ competes with Ca^{2+} for the cation-binding site in bovine oxidase as well as in the D477A mutant of *P. denitrificans* COX. However, whereas two Na^+-binding sites are revealed in bovine enzyme, only one is found in the bacterial oxidase mutant lacking D477. It is proposed that a conserved aspartate D442 (homologous to D477 in *P. denitrificans*) is involved in binding the second Na ion in bovine oxidase.

(4) Careful inspection of the bovine COX crystal structure reveals a potential binding site for the second Na ion ($Na^+_{(2)}$), both in the reduced and oxidized forms of the enzyme. The site is located within 7.4 Å from the first one, $Na^+_{(1)}$, resolved in the crystal structure of COX. The $Na^+_{(2)}$ site involves interaction of the cation with the side-chain oxygens of S441, D442, and D51 in subunit I and also possibly of D50 in subunit I and S205 in subunit II as potential ligands.

(5) Location of the second Na^+-binding site implies direct interaction of sodium ions with the exit part of the so-called H-proton channel in subunit I of bovine oxidase. Whatever the function of the channel, regulation of the H-proton-conducting pathway may provide for a long-sought functional role of the reversible binding of the cations with the mitochondrial cytochrome oxidase.

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