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Kinetic coupling between electron and proton transfer in cytochrome *c* oxidase: Simultaneous measurements of conductance and absorbance changes

(electron transfer/proton transfer/conductometry/electrostatic interaction/flash photolysis)

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ABSTRACT **Bovine heart cytochrome *c* oxidase is an electron-current driven proton pump. To investigate the mechanism by which this pump operates it is important to study individual electron- and proton-transfer reactions in the enzyme, and key reactions in which they are kinetically and thermodynamically coupled. In this work, we have simultaneously measured absorbance changes associated with electron-transfer reactions and conductance changes associated with protonation reactions following pulsed illumination of the photolabile complex of partly reduced bovine cytochrome *c* oxidase and carbon monoxide. Following CO dissociation, several kinetic phases in the absorbance changes were observed with time constants ranging from ≈3 μs to several milliseconds, reflecting internal electron-transfer reactions within the enzyme. The data show that the rate of one of these electron-transfer reactions, from cytochrome a_3 to *a* on a millisecond time scale, is controlled by a proton-transfer reaction. These results are discussed in terms of a model in which cytochrome a_3 interacts electrostatically with a protonatable group, *L*, in the vicinity of the binuclear center, in equilibrium with the bulk through a proton-conducting pathway, which determines the rate of proton transfer (and indirectly also of electron transfer). The interaction energy of cytochrome a_3 with *L* was determined independently from the pH dependence of the extent of the millisecond-electron transfer and the number of protons released, as determined from the conductance measurements. The magnitude of the interaction energy, 70 meV (1 eV = 1.602×10^{-19} J), is consistent with a distance of 5–10 Å between cytochrome a_3 and *L*. Based on the recently determined high-resolution x-ray structures of bovine and a bacterial cytochrome *c* oxidase, possible candidates for *L* and a physiological role for *L* are discussed.**

Cytochrome *c* oxidase is the terminal enzyme of the cellular respiratory chain in mitochondria and some bacteria. It is a membrane-bound protein complex that couples an electron current across the membrane to a proton current in the opposite direction (1) with a H^+/e^- stoichiometry of one (2). High-resolution x-ray structures of both the bacterial enzyme from *Paracoccus denitrificans* (3) and the bovine-heart enzyme (4) have recently been determined. Mitochondrial cytochrome *c* oxidase is a multisubunit protein complex that accommodates a number of cofactors associated with the electron-transfer processes. In the catalytic cycle of cytochrome *c* oxidase, electrons from water-soluble cytochrome *c* are transferred sequentially to copper A (Cu_A), cytochrome *a*, and to the binuclear center consisting of cytochrome a_3 and copper B (Cu_B), where O_2 is reduced to water. Although it has not yet been possible to identify the coupling site(s) of electron transfer to proton transfer across the cytochrome *c* oxidase-containing membrane, experimental results from several research groups point toward the binuclear center as the coupling site (for a review, see refs. 5–9). Studies of protonation reactions associated with the reduction/oxidation of the binuclear center are thus important in the elucidation of the mechanism of proton pumping by the enzyme (cf. refs. 10 and 11).

The so-called flow-flash technique (reviewed in ref. 7) has been used to investigate protonation reactions associated with reduction of dioxygen by partly or fully reduced cytochrome *c* oxidase (12, 13). In this work, we have studied proton uptake and release in the binuclear center following laser-flash photolysis of the carbon monoxide-mixed valence (partly reduced) complex of cytochrome *c* oxidase in the absence of dioxygen. This technique has previously been used to investigate internal electron-transfer reactions (14–18) and makes it also possible to study protonation reactions that are directly coupled to the electron transfers without interference from proton uptake associated with the reduction of dioxygen to water.

With CO bound to the partly reduced enzyme, the apparent reduction potential of cytochrome a_3 is increased and, due to electrostatic interactions with nearby protonatable groups in the presence of reduced cytochrome a_3, their apparent pKs are increased. Consequently, following dissociation of CO, electrons are transferred from cytochrome a_3 and protons are released from the interacting groups. However, the rates of the electron- and proton-transfer reactions are different. First, there is a rapid electron equilibration between cytochromes a_3 and *a*, which results in a 10–15% reduction of cytochrome *a* with a time constant of about 3 μs and an equilibration between cytochromes a_3/a and Cu_A with a time constant of about 50 μs (16–18). These reactions are followed by a slower electron equilibration in which additional electrons are transferred from cytochrome a_3 to *a* on a millisecond time scale (this reaction will be referred to as the ms phase), presumably limited by the proton release. As found both in the bovine (15, 19) and *Rhodobacter sphaeroides* bacterial enzymes (18), both the extent and time constant of this reaction are pH dependent; the time constant increases from about 0.5 ms at pH 6 to about 6 ms at pH 10 and more electrons are transferred at high pH than at low pH (15, 18, 19). This behavior was modeled in terms of electrostatic interactions between the binuclear center and a protonatable group in the vicinity of cytochrome a_3 (18, 19), in contact with the bulk protons through a proton-conducting pathway, which limits the proton-exchange rate with the bulk and, as a consequence, also the rate of the ms-phase electron transfer from cytochrome a_3 to *a* (19). The model predicts that protons should be released upon oxidation of cytochrome a_3 and that there should be a proton uptake upon reduction of cytochrome a_3. These reactions could be measured time resolved using pH-sensitive dyes. However, since protons are released during the anaerobic preparation of the mixed-valence state, it is difficult to

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Abbreviations: Amp, 2-amino-2-methyl-1-propanol; Cu_A, copper A; Cu_B, copper B.

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control the pH in the absence of a buffer, which must be excluded in this type of experiments.

Consequently, in this work we have used a different approach. Conductance changes associated with changes in the amount of mobile charge following flash photolysis of CO from partly reduced cytochrome *c* oxidase were measured with a time resolution of about 100 μs. This method has previously been applied in studies of bacteriorhodopsin (20) and bacterial photosynthetic reaction centers (21). It is not limited to buffer-free solutions because also changes in the protonation state of the buffer result in changes in the number of mobile charges. Indeed, if different buffers are used, which give rise to different changes in mobile charge upon, for example, proton uptake, the choice of buffer can be used to investigate if the measured conductance changes are associated with protons or with other processes (20, 21).

In this work we report for the first time, to our knowledge, direct evidence for a direct kinetic coupling between an individual electron-transfer reaction from/to the binuclear center and a protonation of a group within the enzyme, in the vicinity of this center. Since this type of reaction is essential for the function of cytochrome *c* oxidase, this group(s) is likely to be involved in catalytically important proton-transfer reactions. The experimental technique described here is a method to directly study these reactions.

MATERIALS AND METHODS

Sample Preparation. Cytochrome *c* oxidase was prepared from bovine hearts using the method described by Brandt *et al.* (22). The enzyme was solubilized from mitochondrial membranes using Triton X-100, followed by hydroxyapatite chromatography and exchange of Triton X-100 for 1% (wt/vol) dodecyl D-maltoside (see ref. 13). It was kept in liquid nitrogen until used. Before the measurements, the enzyme was washed several times in the buffer solution used in the measurements using centricon-50 concentrator tubes (Amicon) until negligible amounts of the original buffer (Tris) remained. All other chemicals were of the purest grade available.

The mixed-valence state of cytochrome *c* oxidase was prepared anaerobically essentially as described (23). A modified 1-cm fluorescence cuvette containing oxidized enzyme was evacuated several times on a vacuum line, and filled first with N_2 and then with CO at 105 kPa. The mixed-valence state, characterized by its optical absorption spectrum, was formed after 1–3 hr, depending on pH (23).

Time-Resolved Absorbance Changes. The experimental setup is of local design and has been described previously (15, 24). The monitoring beam, provided by a 250 W halogen lamp, passed through a heat filter and an interference filter (bandwidth 5 nm) before the sample (1.0-cm cuvette) and a double monochromator (Oriel, Stamford, CT) after the sample. Intensity changes were monitored with a photomultiplier tube [Hamamatsu (Middlesex, NJ) model R269 or R712] connected to a current-to-voltage converter (Hamamatsu model C1053, bandwidth 3 MHz), and amplified and filtered using a preamplifier with a variable time constant [Stanford Research (Sunnyvale, CA) model SR560].

Conductance Changes. Conductance changes were measured time resolved using an experimental setup of local design (Fig. 1). Platinum electrodes, placed diagonally in the cuvette and separated by a Teflon spacer, were immersed in the solution. Wires from the electrodes were passed through a rubber septum and connected to the input of a lock-in amplifier (Stanford Research model SR510) as shown in Fig. 1. To improve the time resolution of the setup, the time constant of the built-in low-pass filter was reduced to ≈100 μs by reducing the value of capacitor C414 by a factor of 10 (to 3.3 nF).

Experimental Procedures. Actinic illumination was provided by an Nd-yttrium/aluminum garnet pulsed laser [Quantel (Santa Clara, CA) model YG570] at 532 nm. The pulse width was ≈10 ns and the energy was ≈50 mJ. Data were recorded on a digital oscilloscope (Nicolet model 490). Time constants (τ, 1/e value) were determined from the experimental traces using the SIGMAPLOT software (Jandel, San Rafael, CA).

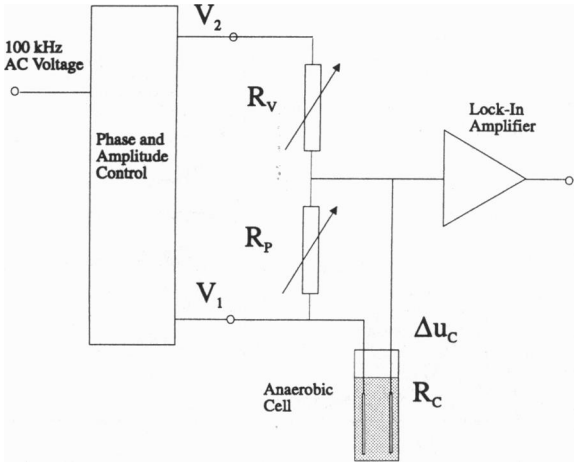

FIG. 1. Schematic view of the experimental setup used for measurements of light-induced conductance changes. The cuvette was connected in parallel with a variable resistor R_P. These together were connected in series with a second variable resistor R_V ($R_V = R_P/2 = R_C/2$). A sinusoidal voltage with a frequency of 100 kHz from an oscillator passed through a circuit which made it possible to vary the phases and relative amplitudes of $V_1(t)$ and $V_2(t)$, applied across the resistor network as shown. Changes in the voltage across the cuvette, Δu_C, were fed into a lock-in amplifier with a gain of 2×10^4.

RESULTS

Following pulsed illumination of the carbon monoxide mixed-valence cytochrome *c* oxidase complex, we observed a rapid increase in absorbance associated with CO dissociation and changes associated with electron transfers from cytochrome a_3 to *a* and Cu_A (see Introduction) with time constants of about 3 μs and 50 μs, respectively, independent of pH in the range 7.4–10 (cf. ref. 16). These changes were followed by a slower increase in absorbance (ms phase, Fig. 2*A*) with a pH-dependent time constant and amplitude (Fig. 3), previously attributed to electron transfer from cytochrome a_3 to *a*, due to interactions with a protonatable group (18, 19). For example, at pH 8.4 the time constant was 2.2 ± 0.3 ms and at pH 10 the time constant was 6.2 ± 0.4 ms. The ms phase was followed by an absorbance decrease that was associated with CO recombination and re-reduction of cytochrome a_3, which at pH 10 was biphasic with time constants of ≈300 ms and ≈800 ms (Fig. 2*B*).

Conductance changes were measured simultaneously with the absorbance changes in the same sample (Fig. 2 *C* and *D*). Absorption of light by the cytochrome *c* oxidase solution gave rise to a rapid (<100 μs) increase in conductance, presumably associated with heating by the laser flash ($<10^{-3}$°C, see below). It was followed by a decrease with a time constant of 6.2 ± 0.7 ms at pH 10, associated with proton release. The conductance then increased with a time constant of ≈300 ms to a level defined by the initial heating change (Fig. 2*D*), followed by a slow (20 s) decrease due to cooling of the sample.

To determine whether the observed conductance changes on the ms time scale were associated with protonation reactions or with exchange of other charges, they were measured in the presence of two different buffers (Fig. 4), glycine (Gly) and 2-amino-2-methyl-1-propanol (Amp), both at pH 9.7 (cf. ref. 21). Assuming that the mobility of the protein is negligible compared with that of the buffer, proton release by cytochrome *c* oxidase and the uptake by the Gly buffer results in a decrease in net mobile charge and thus a decrease in conductance:

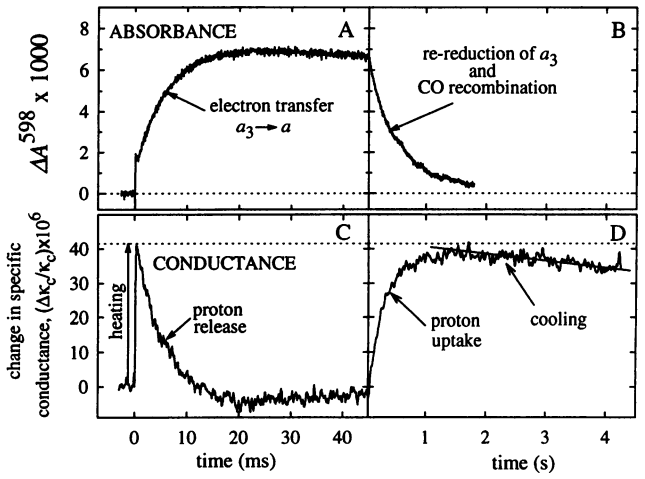

FIG. 2. (*A* and *B*) Absorbance changes at 598 nm following pulsed illumination of mixed-valence cytochrome *c* oxidase. (*C* and *D*) Conductance changes measured simultaneously with the absorbance changes in the same sample with R_V = 727 Ω, κ_C = 5 × $10^{-3}\cdot\Omega^{-1}\cdot dm^{-1}$, V_2-V_1 = 2.3 V. $\Delta\kappa_C/\kappa_C$ was calculated using Eq. **10**. Note the different time scales in *A* and *B*, and *C* and *D*, respectively. Traces in *A* and *C* are averages of 100 traces and in *B* and *D* of 20 traces. The 598-nm wavelength was chosen to minimize the contribution from the CO-dissociation reaction. The concentration of photoactive cytochrome *c* oxidase–CO complexes was determined from the CO-dissociation amplitude at 445 nm using an absorption coefficient of 67 $mM^{-1}\cdot cm^{-1}$ (25). Salt was excluded and a low buffer concentration was used because the measured conductance changes are inversely proportional to the sample conductance. The heating conductance change corresponds to an increase in temperature of $<10^{-3}$°C. Conditions: 0.1% dodecyl-D-maltoside, 5 mM glycine at pH 10, 2.2 μM cytochrome *c* oxidase. The temperature was 21 ± 1°C.

$$(COO^-)CH_2(NH_2) \xrightarrow{+H^+} (COO^-)CH_2(NH_3^+), \quad [1]$$

$$Gly^- \qquad\qquad Gly^0$$

where the superscript of Gly indicates the net charge of the molecule. The same reaction measured in the Amp buffer results instead in an increase in net mobile charge and thus an increase in conductance:

$$(NH_2)(CH_2)C_3H_6OH \xrightarrow{+H^+} (NH_3^+)(CH_2)C_3H_6OH. \quad [2]$$

$$Amp^0 \qquad\qquad Amp^+$$

When measured in the Gly buffer, following flash photolysis of the CO-mixed valence complex of cytochrome *c* oxidase, the initial rapid heating increase in conductance was followed by a decrease on a ms time scale (Fig. 4). When the same measurement was done in the Amp buffer, the rapid increase in conductance was instead followed by a further increase on a ms time scale. This shows that the ms conductance changes were due to proton release by the enzyme.

As shown in Fig. 3*A*, the rates of the proton-release conductance changes and of the ms-phase absorbance changes at 598 nm display the same pH dependencies in the range 8.5–10.5. The amplitude of the absorbance changes increased with increasing pH, reached a maximum around pH 9.5, and then decreased (Fig. 3*B*). The fractions of proton uptake per enzyme molecule at different pH values were calculated using Eqs. **10**, **13**, and **14** (see Appendix) and are summarized in Table 1.

As a control, conductance changes were measured following pulsed illumination of the fully oxidized and the fully reduced cytochrome *c* oxidase in the absence of CO, with the Gly or Amp buffers, and in the fully reduced enzyme with CO present.

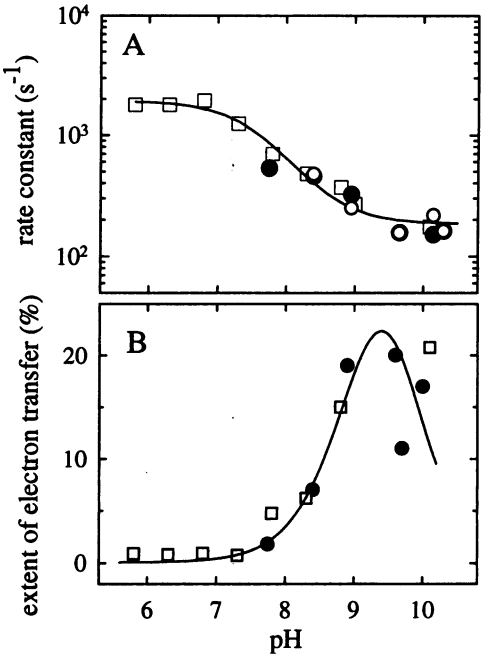

FIG. 3. (*A*) pH dependence of the rates of the ms-phase absorbance changes (●) and of the ms conductance changes (○) shown together with data from Hallén *et al.* (ref. 19; Fig. 4B) (□). The latter were shifted by 0.2 pH units to left. This "offset" is presumably due to different preparations used. (*B*) The fraction of electron transfer from cytochrome a_3 to *a* (calculated using an absorption coefficient of 16 $mM^{-1}\cdot cm^{-1}$ (25) in the ms phase (●) shown together with raw data from Hallén *et al.* (19)† (□).

Under these conditions, no conductance changes associated with protonation reactions were observed.

DISCUSSION

We have simultaneously measured, on a ms time scale, absorbance changes associated with electron transfer between cytochrome a_3 and *a*, and conductance changes associated with proton uptake and release, following flash-induced CO dissociation from partly reduced cytochrome *c* oxidase. As found previously (15, 18, 19), the extent (absorption change amplitude) and rate of the ms-phase electron-transfer reaction were both strongly pH dependent. In addition, we have shown here that changes in conductance take place with the same time constant as the absorbance changes associated with electron transfer between cytochrome a_3 and *a*. Using different buffers, we showed that the conductance changes were associated with proton release by cytochrome *c* oxidase upon oxidation of cytochrome a_3 and proton uptake upon re-reduction.

In the mixed-valence CO complex, electrons are stabilized on cytochrome a_3 and Cu_B by CO, which also stabilizes protons on groups interacting electrostatically with the binuclear center. Upon photodissociation of CO, electrons are transferred from cytochrome a_3 to *a* with a time constant of ≈3 μs, presumably only determined by the electron-transfer parameters (cf. ref. 26) of this reaction (18). Proton transfer from residues interacting with cytochrome a_3/Cu_B is slower and, consequently, electron transfer from cytochrome a_3 to *a* is observed also on a ms time scale, determined by the proton-transfer rate (18, 19).

The pH dependence of the extent of the ms-phase electron transfer between cytochrome a_3 and *a* is explained in terms of a model that is shown in Fig. 5 (18, 19). According to the model,

†The raw data from ref. 19 has been used. The data in figure 4*C* in ref. 19 should not be compared directly to the data from this work because the data in ref 19 were normalized incorrectly.

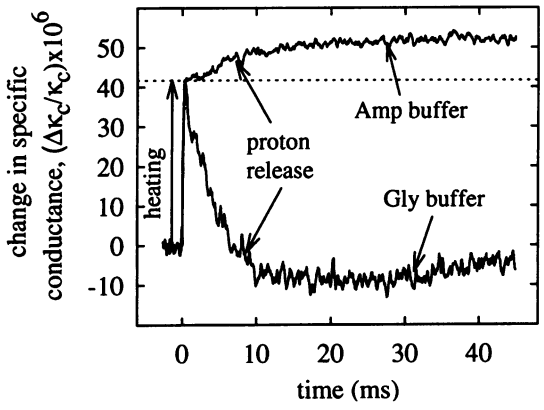

FIG. 4. Conductance changes measured in two different buffers. Upper trace is Amp and the lower trace is Gly. The upper and lower traces are averages of 500 and 50 traces, respectively. For clarity, the data were normalized to the same heating step. Other conditions: 0.1% dodecyl-D-maltoside, 5 mM buffer at pH 9.7, 3.5 μM cytochrome *c* oxidase. The temperature was 21 ± 1°C.

cytochrome a_3 interacts electrostatically with a protonatable group, *L*, in the vicinity. For simplicity, only one single group is assumed to be involved. The electron on cytochrome a_3 is stabilized by the proton on *L*, and vice versa. At high pH, a larger fraction of protons is released and a larger fraction of cytochrome a_3 is oxidized than at low pH (Fig. 3*B*).

The pH dependence of the ms-phase rate was explained in terms of *L* being buried inside the protein, in equilibrium with the solvent only through a proton-conducting pathway (19), consisting of one or more protonatable residues and/or water molecules. Several reasons were presented for the postulation of the proton pathway (19). For example, if *L* was in direct contact with the solvent, assuming spontaneous deprotonation of *L* (see refs. 27 and 28) and no interactions with other residues, a pH-independent proton-release rate, determined by the pK of *L*, would be expected. Alternatively, protons may be transferred from *L* to the acceptor during a collision. In a buffered solution at ≈5 mM, as used in this work, the base of the buffer would be the proton acceptor, and the proton-transfer rate from *L* to the buffer would be rapid (on the order of 10 μs) and pH independent when buffers with pKs close to the pH were used (discussed in refs. 27 and 28). At high pH (or in the absence of a buffer), OH^- would be the acceptor and an increasing rate with increasing pH would be expected.

The existence of a proton pathway in cytochrome *c* oxidase has been suggested previously based on functional studies (29, 30) and has been found in the recently determined high-resolution three-dimensional structures of the bovine heart and *P. denitrificans* enzymes (3, 4). Similar proton-conducting

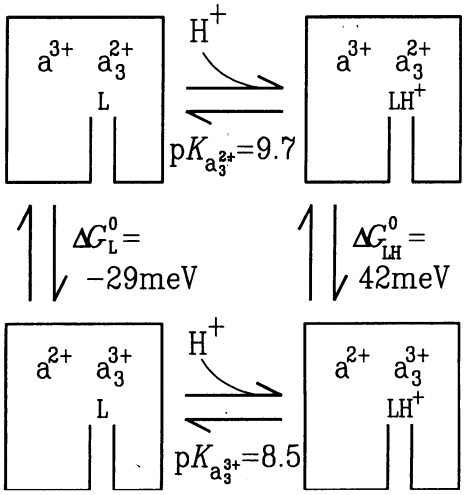

FIG. 5. Model for electrostatic interactions between a protonatable group *L* and cytochrome a_3. The group *L* is in contact with the bulk solution through a proton-conducting pathway that limits the proton-transfer rate. ΔG^0_{LH} and ΔG^0_L are the free energy differences between cytochromes a_3 and *a* with protonated and unprotonated *L*, respectively. The value of ΔG^0_{LH} is from ref. 18.

pathways have also been discussed in bacteriorhodopsin (31) and bacterial photosynthetic reaction centers (32).

According to the model in Fig. 5, the interaction energy between cytochrome a_3 and *L* can be modeled in terms of the change in stabilization energy, ΔG_{La_3}, of the proton on *L* upon reduction of cytochrome a_3:

$$\Delta G^\circ_{La_3} = k_B T \ln(10)(pK_{a_3^{2+}} - pK_{a_3^{3+}}) = k_B T \ln(10)\Delta pK_L, \quad [3]$$

where $pK_{a_3^{2+}}$ and $pK_{a_3^{3+}}$ are the pKs of *L* with cytochrome a_3 reduced and oxidized, respectively. Based on the model in Fig. 5, a relation was derived that relates the pH dependence of the extent of electron transfer in the ms-phase to the pKs of *L* (18). The experimental data in Fig. 3*B* were fit with this model and gave $pK_{a_3^{2+}} \cong 9.7$ and $pK_{a_3^{3+}} \cong 8.5$.

Using the model in Fig. 5, the extent of proton uptake by *L*, ΔH^+_{cal}, can also be calculated from this pK shift and the (measured) fraction of oxidized cytochrome a_3, $\chi(a_3^{3+})$:

$$\Delta H^+_{cal} = \chi(a_3^{3+})\left(\frac{1}{1+10^{pH-pK_{a_3^{2+}}}} - \frac{1}{1+10^{pH-pK_{a_3^{3+}}}}\right). \quad [4]$$

At pH ≪ $pK_{a_3^{3+}}$, *L* is always protonated, independently of the reduction level of cytochrome a_3 and there is no ms-phase electron transfer nor proton release. Similarly, at pH ≫ $pK_{a_3^{2+}}$, *L*

Table 1. Relative conductance changes ($\Delta\kappa_C$) and fraction proton release

Buffer (pK_a)*	pH	$\Delta\kappa_c$, 1 μM† ×10^6	% ΔH^+_{rel}‡	% ΔH^+_{cal}§	% $\Delta H^+_{rel}/\chi(a_3^{3+})$¶
Tris (8.3)	8.4	0.012	2.5	5.6	15
	8.9	0.045	10	13	34
Gly (9.8)	9.6	−0.078	15	16	50
	9.7	−0.082	15	14	71
	10	−0.11	18	9	67

*The pK_as are at 20°C. The activity coefficients have been assumed to be equal to one in the 5 mM buffers used. The values of χ_B (see Eq. **13**) are −1 and +1 for glycine and Tris, respectively.

†The changes in conductance were normalized to those in a 1 μM solution of photoactive cytochrome *c* oxidase–CO complexes.

‡The fractions of protons released by each enzyme molecule were calculated from the measured conductance changes using Eq. **10**, **13**, and **14**.

§The fraction of protons released (i.e., H^+/enzyme molecule) was calculated using Eq. **4** (based on the model in Fig. 5) and the pK values of *L* calculated from the pH dependence of the extent of electron transfer in the ms phase. Note that this method is independent from that described in ‡.

¶The ratio of the fraction of protons released and the fraction of cytochrome a_3 oxidized (i.e., H^+/e^-).

is always unprotonated and no electron transfer nor proton release is observed. Between these pKs of L, both the ms-phase electron transfer and proton release are observed with a maximum number of protons released at

$$\mathrm{pH} = \frac{\mathrm{pK}_{a_3^{2+}} + \mathrm{pK}_{a_3^{3+}}}{2}.$$

The value of this maximum increases with increasing difference between the pKs, $\Delta\mathrm{pK_L}$, and reaches one released proton per enzyme molecule (and about 100% electron transfer) for $\Delta\mathrm{pK_L} > 3$.

Using Eq. **4** (i.e., the model in Fig. 5), the number of protons released by L upon oxidation of cytochrome a_3 at different pH values were calculated and found to be consistent with the extent of proton release measured directly from the observed conductance changes (see Table 1), which suggests that the model used (Fig. 5) is correct.

The pK of L with cytochrome a_3 oxidized was found to be ≈8.5, which may seem to be too high to be physiologically relevant. However, one should note that in the initial state in these experiments both cytochrome a_3 and $\mathrm{Cu_B}$ are stabilized in the reduced state by CO and that during the studied electron- and proton-transfer reactions $\mathrm{Cu_B}$ remains reduced (17). This electron on $\mathrm{Cu_B}$ may stabilize protons on L, increasing its apparent pK. In the more physiologically relevant situation, during turnover, electrons are first transferred to a fully oxidized binuclear center. In this state, the pK of L is expected to be lower as compared with when $\mathrm{Cu_B}$ is reduced. For example, assuming that the electrostatic interaction energy is the same between L and $\mathrm{Cu_B}$ as between L and cytochrome a_3, in the fully oxidized binuclear center L would have a pK of ≈7.3, i.e., shifted by $\Delta\mathrm{pK_L} \cong 1.2$.

Recently, Verkhovsky *et al.* (11) reported results from studies of electron transfer from cytochrome *c* and other electron donors to oxidized cytochrome *c* oxidase. They found that proton uptake coupled to the reduction of the binuclear center may determine the kinetics of the internal electron transfer from cytochrome $a/\mathrm{Cu_A}$ to this center. According to the model, this electron transfer is energetically unfavorable unless protons can stabilize the electron(s) in the binuclear center. These observations are consistent with the model proposed earlier by our research group for the interaction between the binuclear center and a protonatable group in its vicinity (18, 19), and may reflect the same protonation events. Similar observations have also been made by Mitchell and Rich (10) who found that reduction of the binuclear center is accompanied by proton uptake.

The interaction of L with cytochrome a_3 can also be modeled using Coulomb's law (cf. refs. 33–35):

$$\Delta G_{La_3}(d_{La_3}) = \frac{e^2}{4\pi\varepsilon_0}\frac{1}{d_{La_3}\varepsilon(d_{La_3})} \quad [5]$$

$$\varepsilon(d_{La_3}) = 1 + 60(1 - e^{-0.1\cdot d_{La_3}/10^{10}}) \quad [6]$$

where d_{La_3} and $\varepsilon(d_{La_3})$ are the distance and the empirically-determined distance-dependent dielectric constant (36), respectively, between L and cytochrome a_3. Eq. **6** is valid in the range $5\ \text{Å} \le d_{La_3} \le 20\ \text{Å}$ and has an uncertainty in $\varepsilon(d_{La_3})$ of 50% when used to model the protein dielectrics of an arbitrarily chosen protein.

Combining Eqs. **3**, **5**, and **6** gives a relation between the pK shift of L and the cytochrome a_3 to L distance:

$$\Delta\mathrm{pK_L} = e^2(4\pi\varepsilon_0 k_B T \ln(10)\, d_{La_3}\,(1 + 60(1 - e^{-0.1\cdot d_{La_3}/10^{10}})))^{-1}. \quad [7]$$

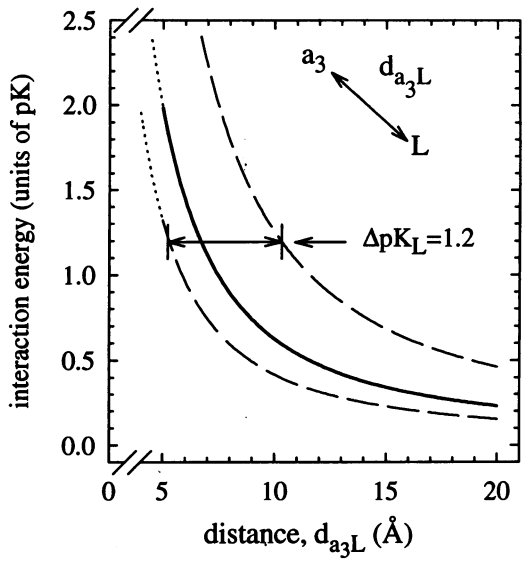

FIG. 6. Electrostatic interaction energy, given in units of pK, between cytochrome a_3 and L, as a function of the distance between these two sites (calculated using Eq. **7**). The dashed lines take into account the error in the dielectric constant (see text). Dotted lines show the curves below the range of validity of Eq. **7** (<5 Å). Possible candidates for L in the relevant distance range are indicated in the text.

In Fig. 6, $\Delta\mathrm{pK_L}$ is plotted as a function of d_{La_3}. Three curves are shown to take into account the uncertainty in $\varepsilon(d_{La_3})$. From the graph, using the experimentally determined $\Delta\mathrm{pK_L}$, it is possible to estimate a range of distances between cytochrome a_3 and possible candidates for L of 5–10 Å, consistent with other experiments that suggest that a group close to the binuclear center may be involved in proton translocation by cytochrome *c* oxidase (24, 37–39). As determined from the recently published high-resolution x-ray structures from *P. denitrificans* (3) and the bovine-heart enzymes (4), possible candidates for L are (su I, *P. denitrificans* numbering is used): E278, Y280, T344, T351, K354, D399, H325 (which may be a $\mathrm{Cu_B}$ ligand), H276, H326 (the His ligands of $\mathrm{Cu_B}$), and the propionate side chains of heme a_3. Several of these groups have been proposed to be directly involved in proton pumping (3, 4). Even though the solution pKs of many of these groups are very different from that of L found in this work, the pK of a group buried inside the protein may be dramatically shifted, as found, for example, in the proton-transfer pathway(s) to the $\mathrm{Q_B}$ site in bacterial photosynthetic reaction centers (reviewed in ref. 32). For simplicity, we have assumed that L is a single protonatable group. However, it is also possible that a cluster of two or more electrostatically interacting groups is involved (see ref. 32). Hopefully, future studies of the ms-phase reaction in mutant forms of cytochrome *c* oxidase from *R. sphaeroides* in which potential candidates for L are modified (40, 41) will make it possible to identify L.

APPENDIX: RELATION BETWEEN FRACTION PROTON UPTAKE AND MEASURED VOLTAGE CHANGE

A small change in the cuvette resistance, ΔR_C, gives rise to a change in the measured voltage, Δu_C (see Fig. 1):

$$\Delta u_C = \frac{(V_2 - V_1)}{4}\frac{R_V \Delta R_C}{R_C^2}, \quad [8]$$

where R_C, R_V, V_1, and V_2 are defined in Fig. 1. Capacitive contributions were found to be negligible for $R_C < 50$ kΩ. Typically, R_C was ≈2 kΩ. The cuvette resistance can be

expressed in terms of the cuvette cell constant, K_C, and the specific conductance, κ_C, and a small change in κ_C is given by:

$$R_C = \frac{K_C}{\kappa_C} \Rightarrow \Delta\kappa_C = -K_C \frac{\Delta R_C}{R_C^2}. \quad [9]$$

In every experiment, R_V and R_P were adjusted so that $R_V = R_P/2 = R_C/2$. This together with Eqs. **8** and **9** gives:

$$\Delta u_C = -\frac{V_2 - V_1}{8} \frac{\Delta\kappa_C}{\kappa_C}. \quad [10]$$

Eq. **10** gives a simple relation between a conductance change and a measured voltage change. The relation was experimentally found to be valid for $\Delta\kappa_C/\kappa_C \leq 0.05$. Typically, we measured changes of $<10^{-4}$.

We will now derive a relation between the number of protons released by the enzyme and a change in conductance. In this work, we have studied conductance changes associated with proton uptake and release in a buffered solution. Consider a buffer that is neutral in charge when it is protonated and negatively charged when it is unprotonated:

$$BH \overset{K_B}{\longleftrightarrow} B^- + H^+.$$

The specific conductance of the buffer solution depends on the concentration of B^-, H^+, OH^-, and all other ions (not involved in protonation reactions) at concentrations C_x (cf. refs. 20 and 21):

$$\kappa_C = \lambda_B[B^-] + \lambda_{H^+}[H^+] + \lambda_{OH^-}[OH^-] + \sum_x \lambda_X C_X$$

$$= \lambda_B \frac{K_B\,[B]_{tot}}{[H^+_{pH}] + K_B} + \lambda_{H^+}[H^+_{pH}] + \lambda_{OH^-}\frac{K_W}{[H^+_{pH}]} + \sum_X \lambda_X C_X, \quad [11]$$

where λ is the molar ionic specific conductances of the ions in units of $\Omega^{-1}\cdot mol^{-1}\cdot dm^2$, $[H^+_{pH}]$ is the concentration of free protons in solution (pH), K_W is the dissociation constant of water, and $[B]_{tot} = [B^-] + [BH]$. We assume that a change in the protonation state of the protein does not affect the conductance because the mobility of the protein is negligible compared with other charged species and that the buffer capacity of the protein is negligible as was found experimentally around pH 8–10.

Differentiation of κ_C with respect to the concentration of protons released by the enzyme, $[H^+_{rel}]$, gives:

$$\frac{\partial\kappa_c}{\partial[H^+_{rel}]} = \frac{\partial\kappa_c}{\partial[H^+_{pH}]}\frac{\partial[H^+_{pH}]}{\partial[H^+_{rel}]}$$

$$= \left(-\lambda_B \frac{K_B[B]_{tot}}{([H^+_{pH}] + K_B)^2} + \lambda_{H^+} - \lambda_{OH^-}\frac{K_W}{[H^+_{pH}]^2}\right)$$

$$\times \left(\frac{([H^+_{pH}] + K_B)^2}{K_B\,[B]_{tot}}\right). \quad [12]$$

The second factor is essentially the inverted buffer capacity of the buffer. Assuming small changes in conductance Eq. **12** gives a relation between a change in conductance and the number of protons released by the enzyme:

$$\Delta[H^+_{rel}] = \kappa_C\left(x_B\lambda_B + \left(\lambda_{H^+} - \frac{\lambda_{OH^-}K_W}{[H^+_{pH}]^2}\right) \times \frac{([H^+_{pH}] + K_B)^2}{K_B\,[B]_{tot}}\right)^{-1}\left(\frac{\Delta\kappa_C}{\kappa_C}\right). \quad [13]$$

The factor χ_B, introduced in Eq. **13**, has a value of -1 or $+1$ in buffers in which the unprotonated form is negative or neutral in charge, respectively. Combining Eqs. **10** and **13**, a measured voltage can be related to the number of protons released. The cell constant of the cuvette used in these experiments was measured and the specific conductance was obtained from:

$$\kappa_C \cong \frac{3.6}{R_V}\,[\Omega^{-1}dm^{-1}] \quad \text{for } R_V < 2k\Omega. \quad [14]$$

Values of R_V were measured in each experiment (e.g., legend to Fig. 2). The value of λ_B for glycin (used in some of the experiments) was determined from a titration of a 5 mM glycin solution with HCl. This and other parameter values are:

$\lambda_B(\text{glycin}) \cong 0.47$, $\lambda_{H^+} = 3.25$, λ_{OH^-}

$= 1.78\,[\Omega^{-1}mol^{-1}dm^2]$, at 20°C.

Typically, V_2-V_1 was 2.3 V.

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