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The mechanism of electron gating in proton pumping cytochrome *c* oxidase: the effect of pH and temperature on internal electron transfer

Peter Brzezinski and Bo G. Malmström

Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Göteborg, Göteborg (Sweden)

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Electron-transfer reactions following flash photolysis of the mixed-valence cytochrome oxidase-CO complex have been measured at 445, 598 and 830 nm between pH 5.2 and 9.0 in the temperature range of 0–25 °C. There is a rapid electron transfer from the cytochrome a_3-Cu_B pair to Cu_A (time constant: 14200 s^{-1}), which is followed by a slower electron transfer to cytochrome *a*. Both the rate and the amplitude of the rapid phase are independent of pH, and the rate in the direction from Cu_A to cytochrome a_3-Cu_B is practically independent of temperature. The second phase depends strongly on pH due to the titration of an acid-base group with $pK_a = 7.6$. The equilibrium at pH 7.4 corresponds to reduction potentials of 225 and 345 mV for cytochrome *a* and a_3, respectively, from which it is concluded that the enzyme is in a different conformation compared to the fully oxidized form. The results have been used to suggest a series of reaction steps in a cycle of the oxidase as a proton pump. Application of the electron-transfer theory to the temperature-dependence data suggests a mechanism for electron gating in the pump. Reduction of both cytochrome *a* and Cu_A leads to a conformational change, which changes the structure of cytochrome a_3-Cu_B in such a way that the reorganizational barrier for electron transfer is removed and the driving force is increased.

Introduction

Cytochrome *c* oxidase, the terminal component of the mitochondrial respiratory chain, is a redox-linked proton pump [1,2]. The operation of such a pump requires transitions between two conformational states, which provide an alternating access of the proton-translocating group to the two sides of the membrane. The coupling of proton translocation to an electron-transfer reaction demands, in addition, that the input and output of electrons take place in different conformations. Such electron gating must be based on a structural control of the rate of electron transfer into and out of the pump redox site [3].

Our research group has previously shown that reduction of both cytochrome *a* and Cu_A, the primary electron acceptors in the oxidase, triggers a conformational transition, which is necessary for rapid electron transfer to cytochrome a_3-Cu_B [4,5]. In this communication we provide additional strong evidence for the involvement of this conformational change in electron gating. This is derived from a study of the pH and temperature dependence of internal electron transfer in the mixed-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Correspondence: B.G. Malmström, Department of Biochemistry and Biophysics, Chalmers University of Technology, 412 96 Göteborg, Sweden.

valence state of the oxidase, i.e., an enzyme form in which cytochrome *a* and Cu_A are both oxidized, whereas cytochrome a_3 and Cu_B are both reduced.

Boelens et al. [6] were the first to demonstrate that on flash photolysis of the mixed-valence oxidase there is a small back-flow of electrons from cytochrome a_3 to Cu_A. We have confirmed this observation but also found a second, slower phase, in which there is electron transfer to cytochrome *a*. The rate constants as well as the equilibrium constant of the first phase are independent of pH. In addition, these constants show a very small temperature dependence. The second phase, on the other hand, becomes most pronounced but slower at high pH because of decrease in the rate constant for back electron transfer from cytochrome *a*. This phase also displays a complex temperature dependence.

On the basis of our results we suggest a detailed reaction sequence for the electron-transfer and proton-translocating steps in the pump cycle of the oxidase. We also demonstrate, on the basis of electron-transfer theory [7], that electron transfer in the conformation of the two-electron-reduced enzyme is rapid because of an increased driving force and a lack of a reorganizational barrier. These facts lead us to propose that reduction of cytochrome *a* and Cu_A effects a conformational transition which changes the structure of the cytochrome a_3-Cu_B pair.

Materials and Methods

Cytochrome oxidase was prepared as described in Ref. 8. The oxidase was dissolved in 0.05 M Hepes buffers, containing 0.167 M K_2SO_4 and 0.5% Tween-80, with different pH values. The mixed-valence state cytochrome oxidase was prepared in a Thunberg cuvette (1.00 cm) at room temperature by incubation of oxidized cytochrome oxidase under a CO atmosphere, in the absence of dioxygen, for several hours. Below pH 6.0 the reduction by CO is too slow [9] and the mixed-valence state was therefore prepared by reduction with two equivalents of NADH and phenazine methosulfate. The mixed-valence state was characterized by its optical spectrum [9].

For the flash photolysis experiments a flash lamp-pumped dye laser (Phase-R model 2100-A) was used. The lasing dye (Rhodamine 6G, Lambda Physik) was dissolved in 99.5% ethanol at a concentration of $1.5 \cdot 10^{-4}$ M. The maximum output was at 590 nm and the linewidth was approx. 40 nm. The duration of each flash was approx. 0.5 μs and the total energy was about 1 J.

A reaction was started by a laser flash. A light beam from a light source passed through the sample and a monochromator, and was finally registrated by the photomultiplier. Between the light source and the cuvette there was a slit. The slit was focused by a lens on a second slit, which was situated in front of the monochromator. The scattered laser light was not focused on the slit and only a negligible part of it reached the photomultiplier. This makes it possible to do optical measurements at the same wavelength as the laser output.

The photomultiplier was connected to ground with a 1.5 KΩ resistor. The input capacitance of the amplifier was 150 pF.

Results

In Fig. 1 we show that a rapid decrease in absorbance occurs at 445 and 830 nm on flash photolysis of mixed valence carboxycytochrome oxidase. We have followed these reactions at 12 pH values in the range 5.2–9.0 but give only three representative results, as both rates and amplitudes are independent of pH in the entire range. The linear second phase in the absorbance changes represents CO recombination, which was found to have a rate constant of 66 ± 5 s^{-1} at 25 °C (the CO concentration was 1 mM). The amplitude of the absorbance change at 445 nm in this phase is 20-times greater than the rapid phase, but the phase can still be resolved because of the large difference in rate. In the calculations of the rate constant for the initial rapid change, corrections for the CO reaction have been made.

The decreasing absorbance change at 445 nm represents heme oxidation, i.e., of cytochrome a_3. It cannot be due to exited-state electron transfer or local heating, as the decay of the vibrational temperature following laser excitation of a heme protein occurs in a few picoseconds [10]. The

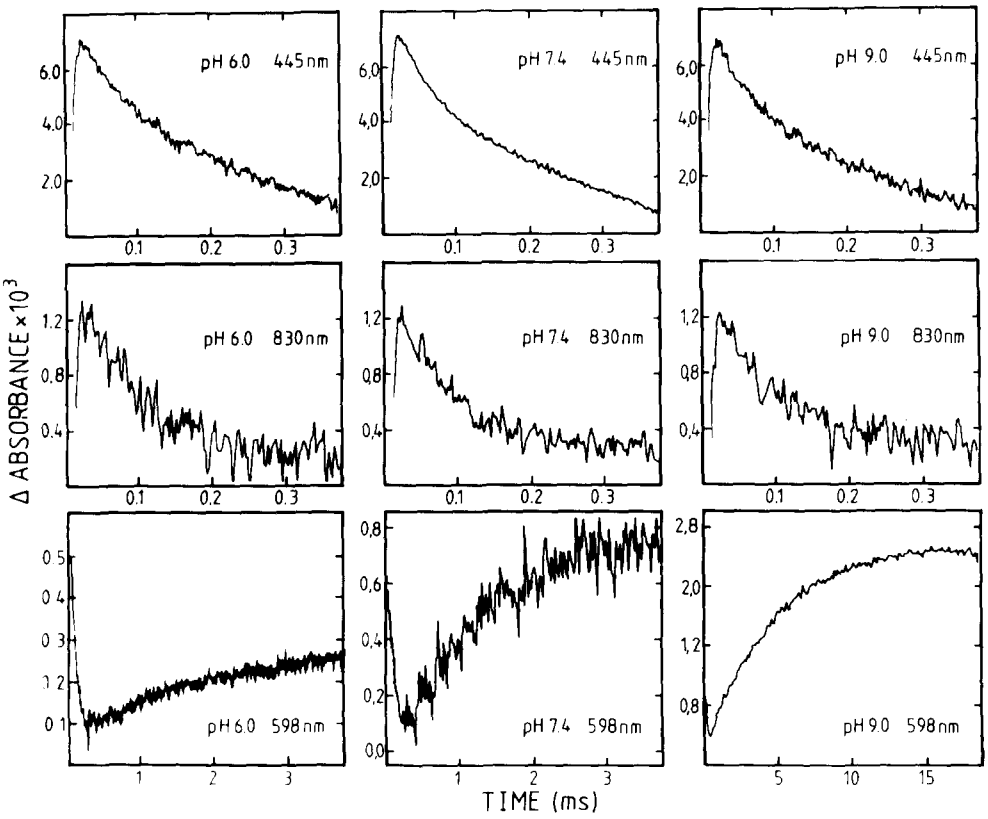

Fig. 1. Absorbance changes at 445, 598 and 830 nm following flash photolysis of the mixed-valence cytochrome oxidase-CO complex at different pH values. Conditions: 2.2 μM (445 nm), 3.6 μM (598 nm) and 11 μM (830 nm) cytochrome oxidase; the buffers contained 0.5% Tween 80/0.167 M K_2SO_4/50 mM Hepes; $pCO = 101$ kPa.

electron acceptor cannot be cytochrome *a*, as the two cytochromes have close to the same difference extinction coefficients at this wavelength [11] so that the absorbance changes would cancel each other out. In addition, data obtained at 605 nm demonstrate that there is no reduction of cytochrome *a*. Instead, the absorbance changes at 830 nm, with the same time constant for the rapid phase, show that the acceptor is Cu_A, in agreement with results of Boelens et al. [6] at pH 7.4. Another possibility would be a primary electron transfer to cytochrome *a*, followed a rapid electron transfer from this cytochrome to Cu_A. This appears less likely, however, as we observe cytochrome *a* reduction in a later, slower phase (Fig. 1, 598-nm data). Thus, we conclude that the rapid phase represents the equilibrium designated with subscript 1 in Fig. 2, with the time constant $(k_1 + k_{-1}) = 14200 \pm 1300\ s^{-1}$ at both wavelengths.

The difference extinction coefficient $\Delta\varepsilon$ (re-

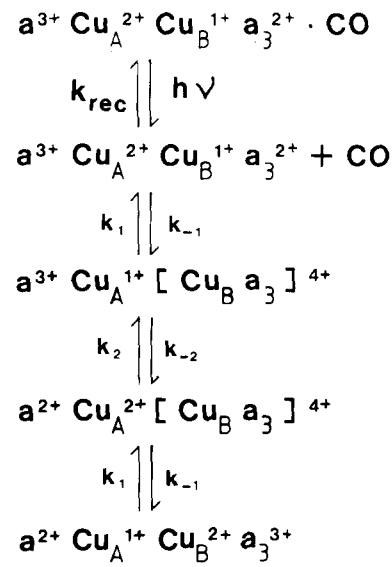

Fig. 2. The electron transfer sequence derived from the experiments in Fig. 1. For explanation, see the text.

duced minus oxidized) for cytochrome a_3 at 445 nm is 80 $mM^{-1} \cdot cm^{-1}$ [6,11]. From this it can be calculated that in saturating laser flashes the amplitude of the rapid phase corresponds to the oxidation of $3.4 \pm 0.8\%$ of cytochrome a_3. The amount of Cu_A reduced, calculated on the basis of $\Delta\varepsilon = 2.3$ $mM^{-1} \cdot cm^{-1}$ at 830 nm [12], is $5.8 \pm 0.8\%$. The fact that more Cu_A is reduced than cytochrome a_3 oxidized indicates that there is a rapid equilibrium between cytochrome a_3 and Cu_B, which is the reason that in Fig. 2 we represent the one-electron reduced binuclear unit as $[Cu_B a_3]^{4+}$. This is consistent with continuous illumination experiments made with the CO-mixed valence complex [12]. Photolysis of carboxycytochrome oxidase at low temperature transfers CO from cytochrome a_3 to Cu_B [13], whereas it would be expected to leave the site at room temperature, in agreement with the rapid redox equilibrium.

The individual rate constants k_1 and k_{-1} in Fig. 2, and the corresponding equilibrium constant, have been calculated from the amplitude and time constant of the rapid phase and are listed in Table I.

The temperature dependence of the rate and equilibrium constants for electron transfer between cytochrome a_3 and Cu_A is shown in Fig. 3. The variation in k_1 with temperature is almost negligible, whereas k_{-1}, and hence the equilibrium constant, displays a small temperature dependence. The relevant thermodynamic parameters are summarized in Table I.

At 598 nm, which is an isosbestic point for the recombination reaction, another slow phase, following the initial rapid absorbance decrease can be observed, as shown in Fig. 1. The amplitude of the rapid change corresponds to the same degree of oxidation of cytochrome a_3 as calculated from the 445-nm data, on the basis of a $\Delta\varepsilon$ for cytochrome a_3 at 598 nm of 4.1 $mM^{-1} \cdot cm^{-1}$ [10]. In the slow phase the absorbance increases, which corresponds to reduction of the heme of cytochrome a, which dominates the absorbance changes at this wavelength. We cannot decide whether this phase represents an equilibrium between the a_3-Cu_B unit and cytochrome a or between Cu_A and cytochrome a, because at 830 nm the absorbance changes due to the recombination reaction dominate in the time range involved. In addition, simulations show that the expected absorbance change at 830 nm due to Cu_A oxidation would be too small to be detected because of the small extent of electron transfer. In Fig. 2 we have, however, depicted Cu_A as the electron donor (reaction depicted by subscripts 2), as it seems likely that there is only one pathway for the internal electron transfer between the two halves of the molecule. In addition, it may be noted that if the donor were cytochrome a_3, then the potential difference between it and cytochrome a should be 175 mV (pH 7.4), which is contrary to other observations [1].

Boelens et al. [6] also observed cytochrome a reduction in continuous illumination experiments

TABLE I

RATE CONSTANTS AND THERMODYNAMIC PARAMETERS FOR STEP 1 IN FIG. 2

(Rate constants and ΔG^O at 25 °C)

Rate		Thermodynamic parameters		
		$\Delta H^{\ddagger}$ ($kJ \cdot mol^{-1}$)	$\Delta S^{\ddagger}$ ($kJ \cdot mol^{-1} \cdot K^{-1}$)	$\Delta G^{\ddagger}$ ($kJ \cdot mol^{-1}$)
Forward (k_1)	13600 ± 1400 s^{-1}	2.1	−0.15	47
Backward (k_{-1})	550 ± 150 s^{-1}	32	−0.077	55
		ΔH^O ($kJ \cdot mol^{-1}$)	ΔS^O ($kJ \cdot mol^{-1} \cdot K^{-1}$)	ΔG^O ($kJ \cdot mol^{-1}$)
Equilibrium constant ($K_1 = k_1/k_{-1}$)	25 ± 5	-29 ± 1	-0.072 ± 0.004	-7.9 ± 0.5

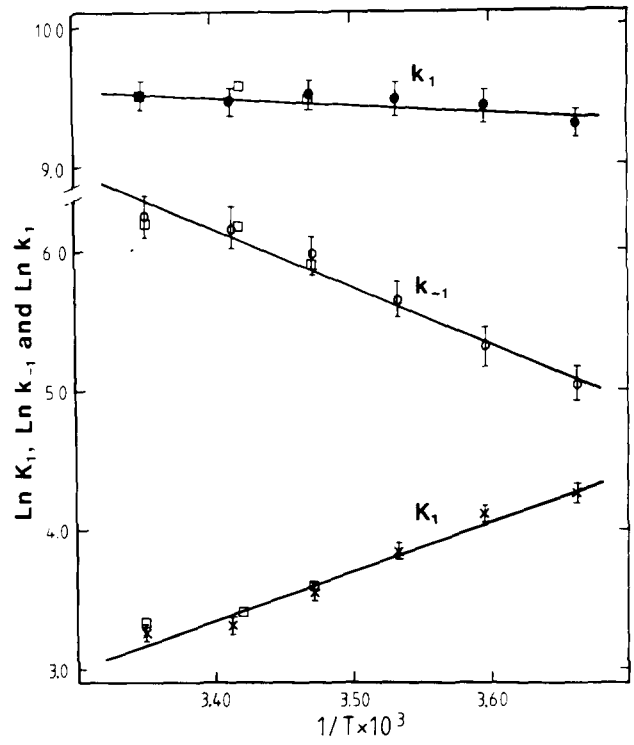

Fig. 3. Temperature dependence of the fast phase in Fig. 1. The rate constants k_1 and k_{-1} are defined in Fig. 2. K_1 is the equilibrium constant, i.e., k_1/k_{-1}. The lines represent linear least-square fits to the data points. Conditions: □, pH 8.0; all other points, pH 6.9.

at high pH, which they ascribed to a decrease in the reduction potential of cytochrome a_3 due to binding of an OH^- ion to the oxidized cytochrome. We have confirmed this, but our slow phase at 598 nm does not represent the same reaction, as the OH^- binding is too slow to interfere except at pH 9.0; in this case a correction has been introduced.

As seen from Fig. 1, the rates and amplitudes of the slow phase at 598 nm depend markedly on pH. The rate constants k_2 and k_{-2} (Fig. 2) as a function of pH at 25°C are shown in Fig. 4, which also gives the corresponding equilibrium constant K_2. The pK_a of the acid whose dissociation regulates K_2 is 7.6 ± 0.2.

The slow phase at 598 nm shows a complex temperature dependence, the rate constant k_2 increasing with temperature between 0 and 10°C and decreasing between 15 and 25°C (data not shown). This probably has no profound theoretical significance but is due to a branched mechanism caused by the fact that electron transfer can occur with different rates in the protonated and unprotonated enzyme forms (see Discussion).

From the amplitudes of the absorbance changes we have calculated the reduction potentials of cytochrome a, cytochrome a_3 and Cu_B at pH 7.4, and 25°C, as shown in Table II. These calculations are based on the assumption that Cu_A has a reduction potential of 260 mv [6] and that there is

TABLE II

POTENTIALS OF THE METAL CENTERS IN E_2 STATE CYTOCHROME OXIDASE

The values are calculated from experimental results at pH 7.4; 25°C (Table I, Fig. 4), assuming a potential of 260 mV for Cu_A.

Center	Reduction potential (mV)
a	225 ± 15
Cu_A	260
Cu_B	355 ± 10
a_3	345 ± 10

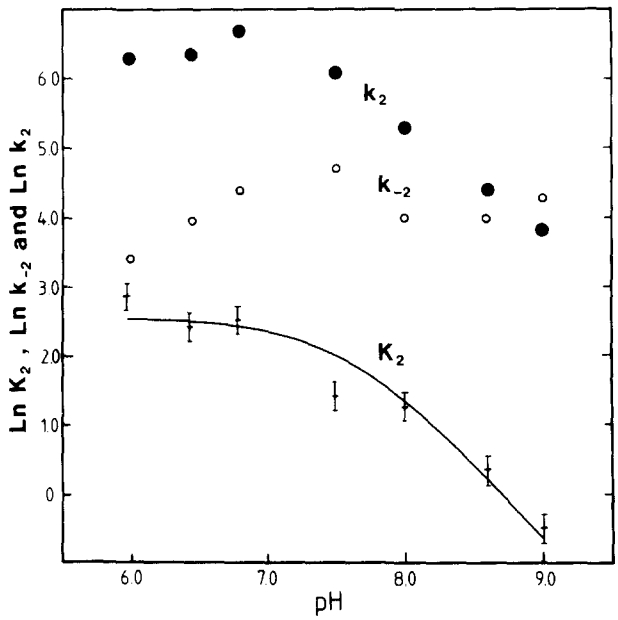

Fig. 4. The pH dependence of the slow phase recorded at 508 nm in Fig. 1. The rate constants k_2 and k_{-2} are defined in Fig. 2. K_2 is the equilibrium constant, i.e., k_2/k_{-2}. The solid curve is calculated on the basis of a decrease in K_2 on the dissociation of one proton from an acid with $pK_a = 7.6 \pm 0.2$. The rate constants (and equilibrium constant) at pH 9.0 are compensated for the binding of OH^- to oxidized a_3.

no potential interactions between cytochrome a_3 and Cu_B (cf. Discussion).

Discussion

Our results have important implications for the mechanism of electron gating in the operation of cytochrome oxidase as a proton pump. To demonstrate this, we start by presenting in Fig. 5 an expanded version of a pump cycle we have formulated earlier [14]. This cycle can be used to simulate the pH dependence of the kinetic behavior of cytochrome *c* and cytochrome *a* in steady-state and transient kinetic experiments under a wide variety of conditions (Thörnström, P.-E., Brzezinski, P. and Malmström, B.G., unpublished results).

The potentials given in Table II show that we are observing a sequence of reactions occurring in the E_2 state, as the potential to cytochrome *a* is 285 mV in the fully oxidized enzyme, E_1, but drops to 220 mV when cytochrome a_3 is reduced [15,16]. Our analysis assumes that there are no redox or spectral interactions. A positive redox interaction between cytochrome a_3 and Cu_B can be excluded, as Cu_A, which is the electron acceptor in our rapid phase, cannot be a two-electron acceptor. Blair et al. [17] have argued for a weak negative interaction, but introducing this does not change our conclusion that we are in a state in which cytochrome a_3 has much higher potential than cytochrome *a*. Significant spectral interactions have been excluded [11,18], even if weak interactions have been noted [19].

The fact that our rapid phase is independent of pH between 5.2 and 9.0 is the reason that we, in Fig. 5B, have let the first proton dissociate before the electron transfer to cytochrome a_3-Cu_B. The pK_a for this proton must be below 5, which is consistent with the transition-state mechanism [2], in which the proton-translocating group must have a low pK_a in both the E_1 and E_2 states. In this case it can be estimated [14] that the off rate for the proton is 10^6 s^{-1}, which is faster even than our rapid phase. It may be noted that to describe the pH dependence of k_{cat} in steady-state experiments [20,21], three pK_a values are necessary, one being 4.5.

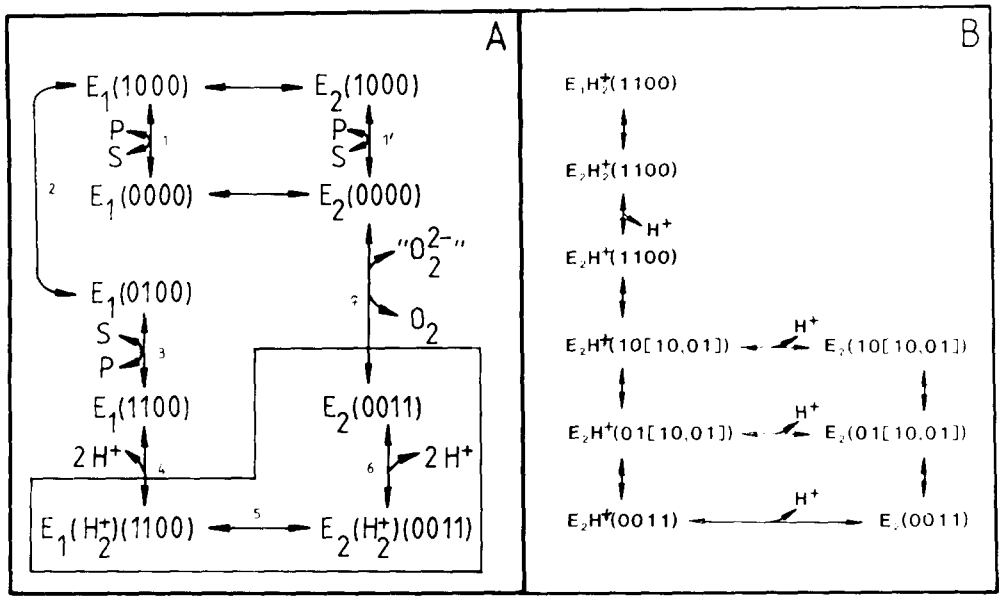

Fig. 5. A reaction cycle for cytochrome oxidase as a proton pump. E_1 and E_2 represent the input and output states of the pump. The digits in parenthesis designate cytochrome *a*, Cu_A, Cu_B and cytochrome a_3 in that order, 0 representing an oxidized center and 1 a reduced one; $E_1(0100)$, for example, stands for a molecule with Cu_A reduced and all other centers oxidized. A is the cycle suggested before the present study. B is an expansion of the enclosed reactions in A, necessary to explain the pH dependence of the electron-transfer sequence in Fig. 2 (here written in the forward direction of the normal catalytic sequence). [10,01] represent the rapid redox equilibrium in $[Cu_B a_3]^{4+}$ (see Results).

To account for the fact that internal electron transfer (step 5 in Fig. 5B) can only occur in E(1100) [4] and for the observed electron/proton stoichiometry in the proton pump [1,2], the enzyme must be doubly protonated in this reaction step. This makes it unlikely that both protons are translocated by the transition-state mechanism, as the concentration of $E_1(H^+)_2(1100)$ at pH 7 would be 10^{-4}-times the concentration of the preceding unprotonated species (see Ref. 2), requiring an improbably high rate constant for step 5. Thus, the translocation of the second proton undoubtedly involves a thermodynamic linkage (membrane Bohr effect). This is supported by the observed pH dependence of the redox equilibrium K_2, our slow phase (Fig. 4), which corresponds to a change in the potential difference between cytochrome *a* and the electron donor of 59 mV per pH unit. The analysis in Fig. 4 favors the suggestion in Fig. 2 that the donor is Cu_A, as a fit of the equilibrium data to a titration curve cannot be made if it were cytochrome a_3-Cu_B. The equilibrium is shifted towards Cu_A as the pH is lowered, which would lead to an increased rate of electron transfer through the cycle. Thus, the middle pK_a (6.8) of the three values used to describe the pH variation in k_{cat} may be related to the pK_a (7.6) controlling K_2 (Fig. 4). The pK_a values derived from k_{cat} have, of course, no simple meaning, whereas our pK_a is a true thermodynamic constant (cf. Ref. 21). The third pK_a (8.0) may then represent the binding of OH^- to cytochrome a_3, observed by Wever et al. [22] and by us, as has been suggested by Wilms et al. [20].

The intramolecular electron transfer in step 5 of Fig. 5A has earlier been shown [4,23] to be rate limiting in the overall reaction. Even our slow electron-transfer step (k_2 in Fig. 4) at pH 7.4 has a rate constant of 400 s^{-1}, which is higher than k_{cat} under the same conditions (detergent, high ionic strength), which is about 50 s^{-1}. Thus, it appears that it is not the rate constant per se but the conformational transition in the first step of Fig. 5B which limits the rate of the overall reaction. This suggests that electron gating involves a structural change triggered by the reduction of both cytochrome *a* and Cu_A (cf. Ref. 5), and we will now support this hypothesis by the applica-

tion of electron-transfer theory [7] to our results.

There are at least four factors that determine the rate of intramolecular electron transfer between two redox centers some distance apart in a protein [3,7]: 1, the driving force, i.e., the free-energy change ΔG^{O}, for the reaction; 2, the reorganizational energy barrier due to different nuclear positions in the oxidized and reduced centers; 3, the distance between the two centers; 4 the intervening medium. It can be shown that at least the two first factors are important to change the slow electron-transfer in E_1 to a rapid reaction rate in E_2. We have already pointed out that cytochromes a and a_3 have the same potential in the E_1 state [15,16], whereas in E_2 there is a potential difference of 120 mV between them and a difference of 85 mV between Cu_A and cytochrome a_3 (Table II). We illustrate this for the rapid electron transfer between Cu_A and cytochrome a_3 with conventional profiles of potential energy surfaces in Fig. 6.

As the driving force for the rapid phase is still modest ($\Delta G^{O} = -7.9$ kJ · mol^{-1}, Table I), the lack of a significant reorganizational barrier in E_2 is even more important for the high rate. This follows from the extremely small temperature dependence of k_1 (Fig. 3). According to Marcus and Sutin's theory [7], the following equations apply to intramolecular electron transfer:

$$\Delta G^{*} = \tfrac{1}{4}\lambda\left(1 + \frac{\Delta G^{O}}{\lambda}\right)^{2} \quad (1)$$

$$E_a \approx \Delta H^{*} \approx \frac{\lambda}{4} + \frac{\Delta H^{O}}{2}\left(1 + \frac{\Delta G^{O}}{\lambda}\right) - \frac{(\Delta G^{O})^{2}}{4\lambda} \quad (2)$$

where λ is the reorganizational barrier, E_a the Arrhenius activation energy, ΔG^{*} the free energy of activation, ΔH^{*} the enthalpy of activation and ΔG^{O} the free energy of reaction.

By application of Eqns. 1 and 2 to the data in Fig. 3 and Table I, it can be shown that ΔG^{*} is close to zero (0.063 kJ · ml^{-1}), because the ratio $\Delta G^{O}/\lambda$ in Eqn. 1 is approx. -1 ($\lambda = 6.7$ kJ · mol^{-1}). We illustrate also the removal of reorganizational barrier in the transition $E_1 \rightarrow E_2$ with the potential energy profiles in Fig. 6. They show as well why the rate constant (k_{-1}) for the back reaction displays a slight temperature dependence, as in this direction $\Delta G^{O} = 7.9$ kJ · mol^{-1}. On the

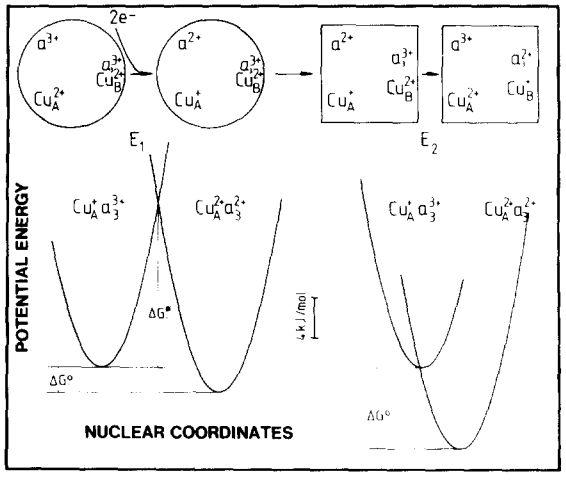

Fig. 6. Schematic illustration of the removal of the reorganizational barrier after transition from the E_1 to E_2 state. The transition is induced by reduction of both cytochrome a and Cu_A. The rapid electron transfer between Cu_A and cytochrome a_3 can be interpreted in terms of potential energy surfaces. ΔG_1^{O} is calculated from E^{O} (Cu_A) = 260 mV and E^{O} (a_3) = 285 mV [15,16]. ΔG_2^{O} is calculated from the potentials listed in Table II. The reorganizational barrier λ in the E_2 state is calculated from experiments (see text). The barrier in the E_1 state is assumed to be larger than in E_2.

basis of a comparison of our results with known structural features we would like to suggest that the reorganizational barrier in E_1 is located in the cytochrome a_3-Cu_B site. It is removed in E_2 by a structural change induced by the reduction of cytochrome a and Cu_A, as illustrated schematically in Fig. 6. In the oxidized enzyme the Cu-Fe distance in the cytochrome a_3-Cu_B site is 3.0–3.5 Å, according to EXAFS measurements [24,25]. On reduction it must increase to about 5 Å to be able to accomodate dioxygen as a μ-peroxo bridge, as evidenced by studies of intermediates in dioxygen reduction [26]. A distance of 5 Å has also been estimated from the zero-field splitting of the triplet state in the complex with NO [27]. The structural change caused by the reduction of both cytochrome a and Cu_A is, in an allosteric fashion, transmitted to the cytochrome a_3-Cu_B site, changing the metal-metal distance. As this site in its oxidized state now has the structure it should have in the reduced state, there is no barrier for electron transfer. When dioxygen binds to the reduced cytochrome a_3-Cu_B site, the driving force increases even more. This does not, however, move

the system into the inverted region [7], as the nuclear coordinates will also change.

The model just presented is supported by work on the cyanide inhibition of cytochrome oxidase [28,29]. This has demonstrated that oxidized cytochrome a_3 in $E_1(0000)$ binds cyanide extremely slowly, whereas there is a very fast binding in $E_1(1100)$ [5]. Thus, reduction of cytochrome a and Cu_A induces a structural change in the cytochrome a_3-Cu_B site, making this available for rapid interactions with ligands.

For long distance electron transfer in a protein, where the donor-acceptor electronic coupling is weak, the rate falls off exponentially with distance (r) between donor and acceptor:

$$k = k_0 \, e^{-\beta r} \qquad (3)$$

where β reflects the extent of electronic coupling through the protein medium [7,30]. The nuclear frequency factor k_0 is commonly assumed to be 10^{13} s^{-1} [7,30] and a recent estimate of β is 1.4 $Å^{-1}$ ([7,30]; Gray, H.B., personal communication). Applying Eqn. 3 to k_1 (Table I) with these parameters, we obtain a distance r of 15 Å between Cu_A and cytochrome a_3-Cu_B. Spectroscopic measurements [31–33] have suggested that 10 Å $< r <$ 20 Å, supporting the validity of the parameters used in Eqn. 3 for long distance electron transfer in proteins.

A similar situation to that described here has been found by Chamorovsky et al. [34] for electron transfer from cytochromes to the reaction center in a photosynthetic system. It was shown that a slow conformational change preceded a rapid electron transfer, whose rate was independent of temperature. This reaction has also been analysed in terms of electron-transfer theory [35].

Bickar et al. [36] have recently proposed that the turnover rate of cytochrome oxidase is governed not by the rate constants for internal electron transfer, which they assume to be large, but by the redox equilibrium, which favors reduced cytochrome a and oxidized a_3. Their mechanism is formally identical with the one in Fig. 5A except that it does not include two enzyme states, E_1 and E_2, as required for a proton pump. Our analysis shows that their hypothesis is only partly true. In the E_1 state the conditions for electron transfer are unfavorable, but not in the E_2 state, as illustrated in Fig. 6.

A mechanism of electron gating involving structural changes in Cu_A has been advocated by Blair et al. [3,37]. They suggest that a ligand on Cu_A dissociates on reduction and binds a proton. They furthermore postulate that in this state only can there be a rapid electron transfer from Cu_A to cytochrome a_3-Cu_B. Such a mechanism of electron gating is, however, made unlikely by our results, as Cu_A has the same EPR spectrum, and hence the same coordination, in the mixed-valence state as in the fully oxidized enzyme [38,39].

A direct mechanism of coupling between electron transfer and proton translocation, in which the redox site provides the proton-binding group, is intuitively appealing. Our analysis suggests, however, that the coupling is more indirect. A redox-induced structural change, which is transmitted from one half of the pump protein to the other half in an allosteric fashion, is rapid only in the protonated state of the protein. In a sense, this is a disappointing conclusion, because it makes it very difficult to progress from the schematic model in Fig. 6 to a detailed molecular picture. This requires a high-resolution three-dimensional structure of cytochrome oxidase in both the E_1 and E_2 states to be made available, but there appears to be little hope that this will become a reality in the near future.

Acknowledgements

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