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The effect of pH and temperature on the reaction of fully reduced and mixed-valence cytochrome *c* oxidase with dioxygen

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The reaction of fully reduced and mixed-valence cytochrome oxidase with O_2 has been followed in flow-flash experiments, starting from the CO complexes, at 428, 445, 605 and 830 nm between pH 5.8 and 9.0 in the temperature range of 2–40 °C. With the fully reduced enzyme, four kinetic phase with rate constants at pH 7.4 and 25 °C of $9 \cdot 10^4$, $2.5 \cdot 10^4$, $1.0 \cdot 10^4$ and 800 s^{-1}, respectively, are observed. The rates of the three last phases display a very small temperature dependence, corresponding to activation energies in the range 13–54 kJ · mol^{-1}. The rates of the third and fourth phases decrease at high pH due to the deprotonation of groups with pK_a values of 8.3 and 8.8, respectively, but also the second phase appears to have a small pH dependence. In the reaction of the mixed-valence enzyme, three kinetic phases with rate constants at pH 7.4 and 25 °C of $9 \cdot 10^4$, 6000 and 150 s^{-1}, respectively, are observed. The third phase only has a small temperature dependence, corresponding to an activation energy of 20 kJ · mol^{-1}. No pH dependence could be detected for any phase. Reaction schemes consistent with the experimental observations are presented. The pH dependencies of the rates of the two final phase in the reaction of the fully reduced enzyme are proposed to be related to the involvement of protons in the reduction of a peroxide intermediate. The temperature dependence data suggest that the reorganization energies and driving forces are closely matched in all electron transfer steps with both enzyme forms. It is suggested that the slowest step in the reaction of the mixed-valence enzyme is a conformational change involved in the reaction cycle of cytochrome oxidase as a proton pump.

Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*-oxygen oxidoreductase, EC 1.9.3.1), the terminal enzyme of cellular respirationn in eukaryotic organisms and in some bacteria, is a redox-linked proton pump [1]. Our research group has previously suggested [2–4] that the coupling between the electron-transfer reaction and the proton translocation is related to the internal electron transfer from the primary electron acceptors, cytochrome *a* and Cu_A, to the binuclear dioxygen-reducing site, cytochrome a_3-Cu_B. To test this hypothesis, it becomes important to study the effect of pH on the rate of the internal electron transfer in various states of the enzyme. It has furthermore been argued [3,5] that the structural control of this rate in electron gating [6] involves changes in the nuclear reorganization energy [7]. In view of this, the effect of temperature on the rate also assumes considerable importance.

We have earlier reported [3] the effect of pH and temperature on the internal electron transfer in the mixed-valence oxidase under anaerobic conditions. In this paper we describe such effects on the reaction of dioxygen with the fully reduced and the mixed-valence enzyme. The results of previous studies of these reactions, usually at constant pH and temperature, by flow-flash spectrophotometry have recently been reviewed [8,9]. Important information about the nature of the intermediates has also been derived by resonance Raman techniques [10,11].

Our flow-flash measurements with the fully reduced oxidase confirm that there are three successive kinetic phases [8,9], but we have also detected an initial rapid reaction (about 10^5 s^{-1}) previously observed only by time-resolved resonance Raman spectroscopy [10]. On the basis of our results, we propose that an oxygen

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; TMPD, tetramethyl-*p*-phenylenediamine.

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adduct of reduced cytochrome a_3 is first formed, and it is then rapidly converted to a peroxide by electron transfer from both cytochrome a_3 and Cu_B. The effect of pH on the rates suggests that the peroxide intermediate can receive a third electron, presumably from Cu_A, only if it is first protonated. The rate of the final step also shows a significant pH dependence. All three electron-transfer steps display a very small temperature dependence, even if the largest one is associated with the slowest step, which probably involves a breaking of the O–O bond. It is argued that the enzyme is in a state in which the reorganization energy of the two rapid electron-transfer steps is largely balanced by the driving force [7].

Our results with the mixed-valence oxidase confirm the earlier finding [12] that three successive kinetic phases can be resolved in the reaction with O_2. Like the other investigators [12] we assign the initial phase to the formation of an O_2 adduct of reduced cytochrome a_3, whereas we propose that the intermediate phase involves the formation of a peroxide intermediate at the binuclear site. This is much slower than the corresponding reaction starting from the fully reduced enzyme [8,9], indicating that the reactions at the binuclear site are influenced by the redox state of the other sites. The rate of reaction in the intermediate phase, as well as that of the final slow phase, shows no or a very small dependence on both pH and temperature.

Materials and Methods

Cytochrome oxidase from bovine heart was prepared as described in Ref. 13. It was dissolved in 50 mM Hepes or Mes buffers, depending on the pH, containing 0.167 M K_2SO_4 and 0.5% Tween-80. Cytochrome *c* from horse hearts was isolated by the procedure of Brautigan et al. [14] and then further purified by ion exchange chromatography.

The fully reduced oxidase-CO complex was prepared in a stopped-flow storage syringe under an O_2-free atmosphere in the presence of 100 nM cytochrome *c*, 10 μM TMPD and excess ascorbate. It was rapidly mixed with oxygenated buffer in a flow-flash apparatus with a computer-controlled ram unit to push the syringes. The solutions in the storage syringes were kept under positive gas pressure. Injection syringes of unequal sizes were used to give a mixing ratio of O_2-buffer to oxidase-CO of 4:1, yielding an O_2 concentration of approx. 1 mM in the cuvette. The optical path of the cuvette was 1 cm. Monochromatic analysis light was employed in combination with a monochromator in front of the photomultiplier. The mixed-valence cytochrome oxidase was prepared by incubation of the oxidized enzyme in a stopped-flow storage syringe under an O_2-free CO atmosphere.

For the photolysis a laser (Phase-R model 2100-A) with a lasing dye (Rhodamine 6G, Lambda Physik) was used. Its maximum output was at 590 nm, the linewidth was approx. 40 nm and the total energy about 1 J. The time constant of the detecting system was limited by the duration of the flash, which was approx. 1 μs. For some experiments, a new laser (Nd-YAG laser from Quantel) was used. Its output wavelength was 532 nm, the duration of the pulse was 9 ns and the total energy about 0.3 J. The time constant was limited to 0.2 μs by the preamplifier to the photomultipliers. No differences were found in the kinetic parameters determined with the two lasers.

The kinetic traces recorded were fitted to a sum of exponentials in a personal computer with the software Matlab. Such a procedure is associated with appreciable errors [15], which introduces considerable uncertainty in the rate constants and amplitudes, particularly for overlapping kinetic phases (see Results).

Results

The absorption changes at 445 nm during the reaction between fully reduced cytochrome oxidase and O_2 can be resolved into four phases, as shown in Fig. 1. We have analyzed the effect of pH in the range 5.8–9.0 and of temperature from 2 to 40 °C on these phases.

The initial phase, F_1, consists of an absorbance decrease with a rate constant of $9 \cdot 10^4$ s^{-1}. We have not found any significant dependence on pH or temperature of this constant, but the experimental uncertainty is large for this phase. The amplitude of this kinetic component at 445 nm is about 40% of the total amplitude. F_1 is not detectable at 428 or 830 nm, and it could not be observed at 605 nm, since the photomultiplier was saturated from the laser flash during the first 20 μs.

The second phase, F_2, observed at 445 nm (Fig. 1) displays both pH and temperature dependence, as shown in Fig. 2. The rate constant at pH 7.4 and 25 °C is about $2.5 \cdot 10^4$ s^{-1}. F_2 is the first detectable phase at 428 nm, as seen in Fig. 3, and also at 605 nm (data not shown). It can also be observed at 830 nm, as shown in Fig. 4. The component amplitude of F_2 at 445 nm is about 50% of the total amplitude.

The next phase, F_3, is associated with an absorbance increase at 445 nm, but as it overlaps with F_2, it is seen as a plateau or a bump (Fig. 1). It also depends on pH and temperature (Fig. 2). The rate constant at pH 7.4 and 25 °C is about $1.0 \cdot 10^4$ s^{-1}, and the amplitude at 445 nm (with an opposite sign compared to the other phases) is about 25% of the total amplitude. At 605 nm, this phase is also seen as an absorbance increase, whereas there is a decrease at 428 nm (Fig. 3) and 830 nm (Fig. 4).

The fourth and slowest phase, F_4, shows a pH dependence, and it has the strongest temperature dependence

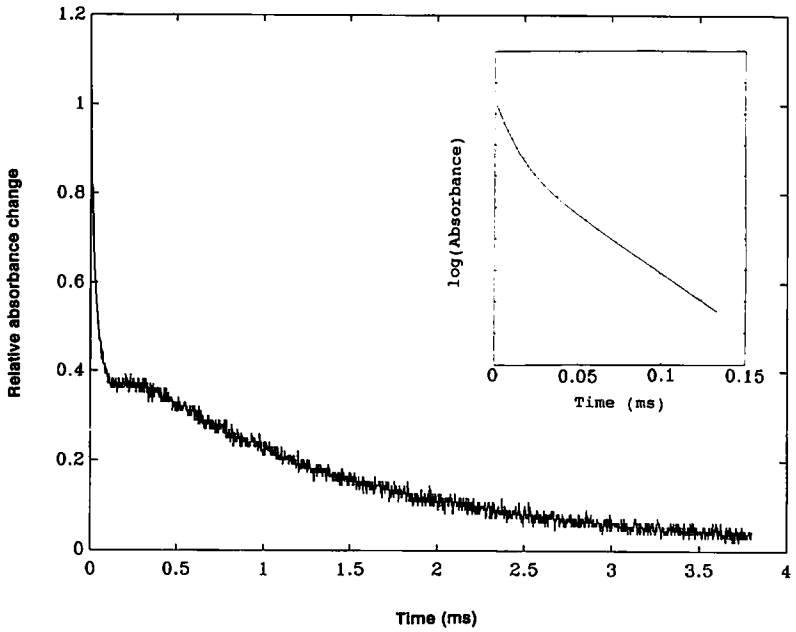

Fig. 1. Absorbance changes at 445 nm following flash photolysis of the fully reduced cytochrome-CO complex in the presence of O_2. The inset gives the initial rapid change on a logarithmic scale, showing that this consists of two exponential components. Conditions: 3 μM cytochrome oxidase and approx. 1 mM O_2 (pH 7.4) and temperature 25 °C.

(Fig. 2). The rate constant at pH 7.4 and 25 °C is about 800 s^{-1}, and the amplitude at 445 nm constitutes about 35% of the total amplitude. As seen in Fig. 3, at 428 nm F_4 is associated with an isosbestic point at pH 9, but it can be observed at lower pH values at this wavelength.

The component amplitudes for all four phases varied

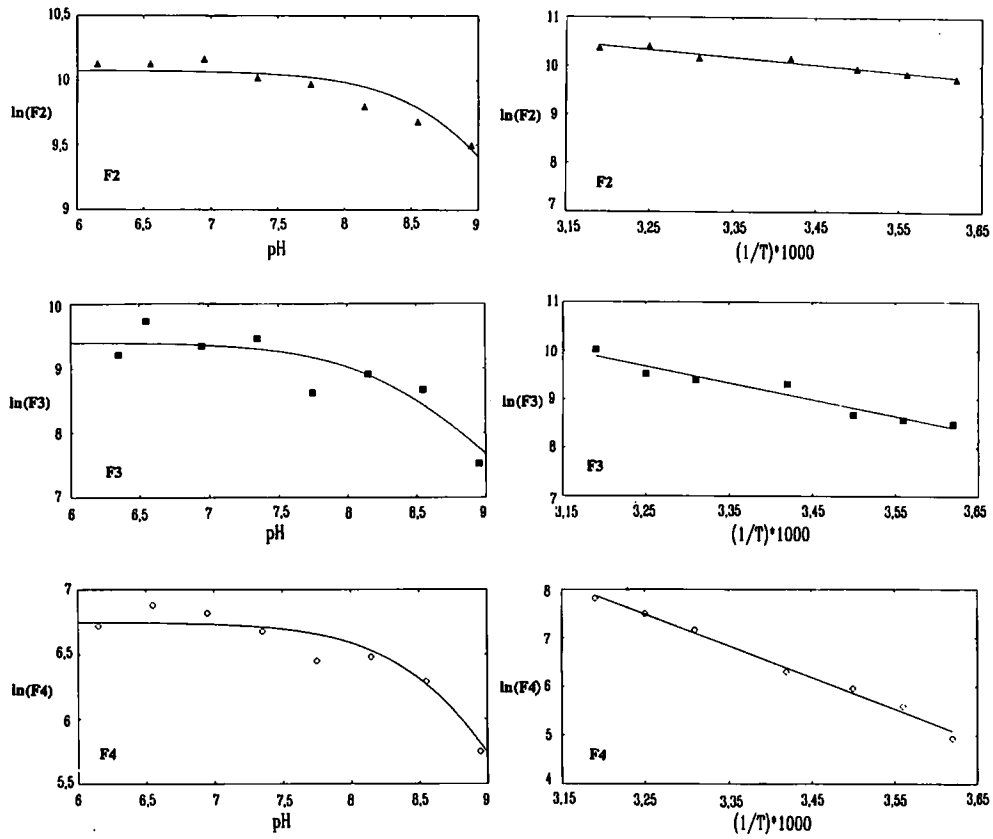

Fig. 2. The effect of pH at 25 °C and the temperature at pH 7.4 on the rate constants for phases F_2, F_3 and F_4. The solid pH curves have been calculated on the basis of a decrease in the rate constants on dissociation of one proton from acids with pK_a values of 9.0, 8.3 and 8.8, respectively. The slopes of the temperature curves give activation energies of 13, 28 and 54 kJ·mol^{-1}, respectively.

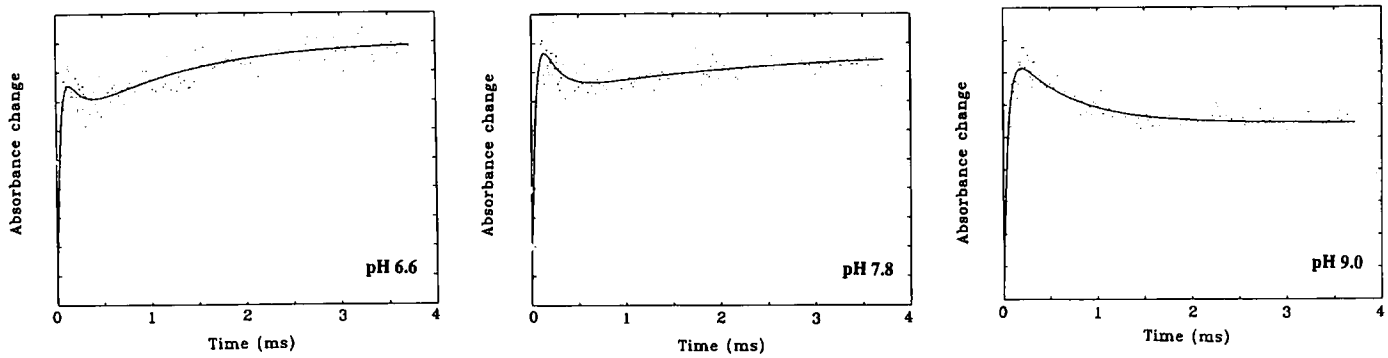

Fig. 3. Absorbance changes at 428 nm following flash photolysis of the fully reduced cytochrome oxidase-CO complex in the presence of O_2 at pH 9.0, 7.8 and 6.6. Other conditions as in Fig. 1.

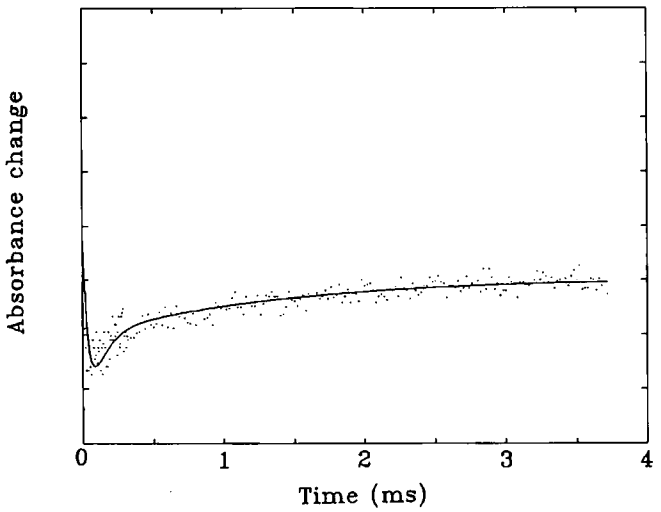

Fig. 4. Absorbance changes at 830 nm following flash photolysis of the fully reduced cytochrome oxidase-CO complex in the presence of O_2 at pH 7.4 and 25 ° C. The oxidase concentration was 10 μM.

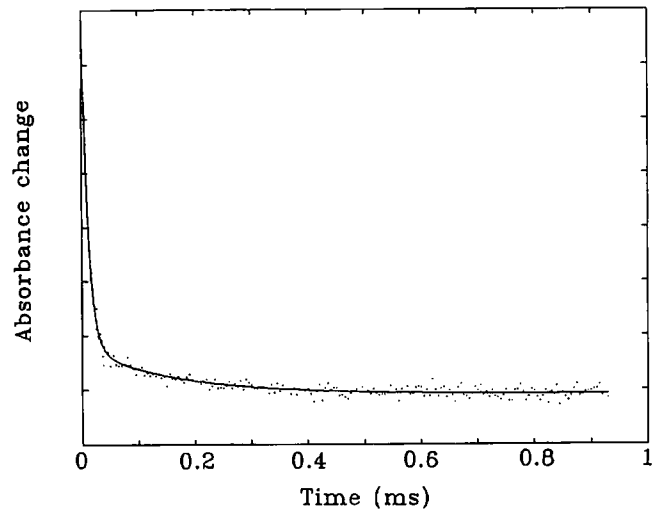

Fig. 5. Absorbance changes at 445 nm following flash photolysis of the mixed-valence cytochrome oxidase-CO complex in the presence of O_2. Conditions: 3 μM cytochrome oxidase and approx. 1 mM O_2 (pH 7.4) and temperature 25 ° C.

with pH, but because of the uncertainties in the curve fitting we have not analyzed these variations in detail.

Application of transition-state theory to the temperature data in Fig. 2 allows the calculation of the entropy of activation, which was found to be -120, -70 and $-55\ J \cdot K^{-1} \cdot mol^{-1}$ for F_2, F_3 and F_4, respectively.

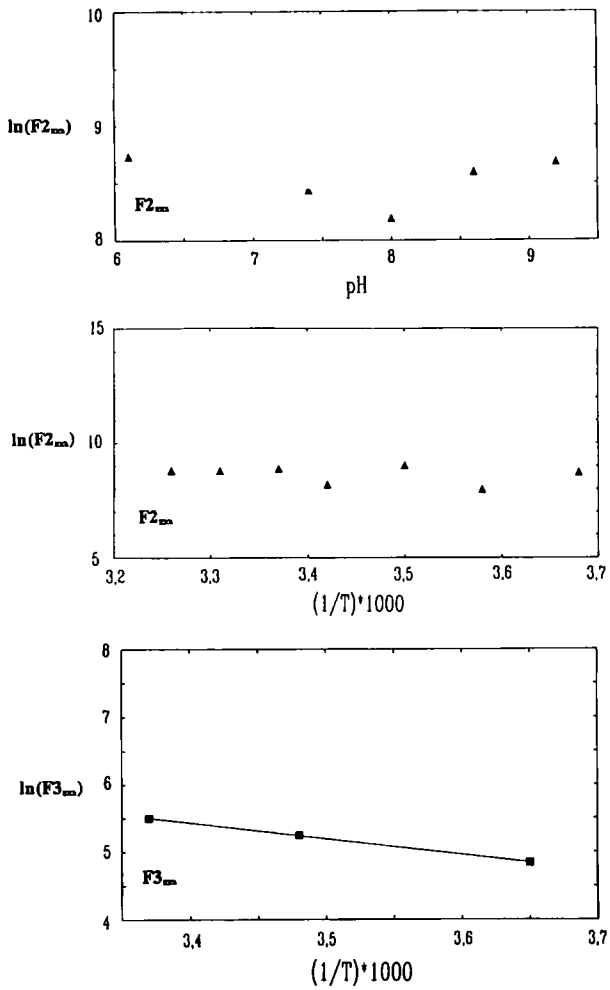

Fig. 6. The effect of pH at 25 ° C and of temperature at pH 7.4 on the rate constants for phases F_{2m} and F_{3m}. The slope of the temperature curve for F_3 corresponds to an activation energy of 20 $kJ \cdot mol^{-1}$.

The reaction between mixed-valence cytochrome oxidase and O_2 displays a biphasic absorption decrease at 445 nm, as shown in Fig. 5. The same two phases, with the same rate constants as determined at 445 nm, can also be observed at 605, where, in addition, a third phase appears [12]. At 428 and 830 nm, one fast phase only could be resolved. We have analyzed the effect of pH in the range 6.2–9.0 and of temperature from 2 to 35 °C on these phases.

The initial phase, F_{1m}, was found to have a rate constant of about $9 \cdot 10^4$ s^{-1}, when determined at 445 or 605 nm. A significantly smaller value ($6 \cdot 10^4$ s^{-1}) was obtained at 428 nm, which may indicate that the reaction observed at the other wavelength is actually biphasic but with rate constants differing by less than a factor of 2. At 445 nm, the amplitude of F_{1m} comprises 90% of the total amplitude. We could not detect any significant dependence on pH or temperature of F_{1m}.

The second phase, F_{2m}, at 445 nm has a rate constant of about 6000 s^{-1} at 25 °C and pH 7.4. The effect of pH and temperature on this rate constant is negligible, as shown in Fig. 6. F_{2m} can also be observed at 605 nm but not at 428 or 830 nm.

The third phase, F_{3m}, seen as an absorbance decrease around 610 nm, has a rate constant of about 150 s^{-1} at pH 7.4 and 25 °C. Also this rate constant displays a very small dependence on pH and temperature, even if the temperature dependence is somewhat larger than that of the other two phases.

From transition-state theory, the entropies of activation were calculated to be -180 and -130 $J \cdot K^{-1} \cdot mol^{-1}$ for F_{2m} and F_{3m}, respectively.

Discussion

Our results agree with the original flow-flash investigations by Gibson and Greenwood [16,17] of the reaction of fully reduced cytochrome oxidase with O_2 and also with subsequent studies by other investigators [8,9]. The initial, very rapid phase had, however, not previously been seen in flow-flash spectrophotometry experiments, since it can only be resolved at high concentrations of O_2. The plateau or bump at 445 nm (Fig. 1) and the absorbance decrease at 428 nm (Fig. 3) associated with our third phase was not observed in the early experiments [16,17], because this feature also manifests itself only at high O_2 concentrations (about 1 mM), as first demonstrated by Brunori and Gibson [18].

In Fig. 7 we give a model for the reaction of fully reduced cytochrome oxidase with O_2, based on our results as well as on other observations summarized in recent reviews [8,9,19]. It should be stressed, however, that it is not possible to assign the absorbance changes in the successive phases to the oxidation or reduction of specific redox centers, because of the unknown spectral contributions of intermediates. This has the consequence that the structures of the intermediates proposed in Fig. 7 must be viewed with considerable reservation.

The first step is most likely the formation of an O_2 adduct of reduced cytochrome a_3. From experiments at low O_2 concentrations [16,17,20], the second-order rate constant has been estimated to be $1 \cdot 10^8$ $M^{-1} \cdot s^{-1}$, which with our O_2 concentration should give a pseudo-first-order rate constant of about 10^5 $M^{-1} \cdot s^{-1}$, in good agreement with our observations. The structural assignment is also supported by time-resolved resonance Raman measurements, which show that a photolabile transient is formed in 40 μs [10]. The lack of a temperature dependence of this phase is probably apparent, since we have not corrected for the increase in the O_2 concentration at low temperatures, which would counteract a decrease in rate.

The second phase we ascribe to the formation of a peroxide intermediate with a concomitant oxidation of cytochrome a_3 and Cu_B, in agreement with many other investigators [8,9,18]. We have not included a proton in this step in Fig. 7, despite the observed pH dependence (Fig. 2). One reason for this is the great uncertainty in the curve fitting for this phase, as its rate constant differs by a factor less than 3 from the following phase. In addition, it appears difficult to suggest a molecular basis for a pH effect on this step. On the other hand, the rate constant is lower than one would expect for electron transfer to O_2 bound in a reduced binuclear metal center [7]. This could indicate that the step is associated with some structural rearrangement, which could be pH dependent. The very small temperature dependence, however, speaks against any large structural change.

As first proposed by Gibson and Greenwood [21] we assume that the third phase is associated with electron transfer from Cu_A to the binuclear center. On the basis

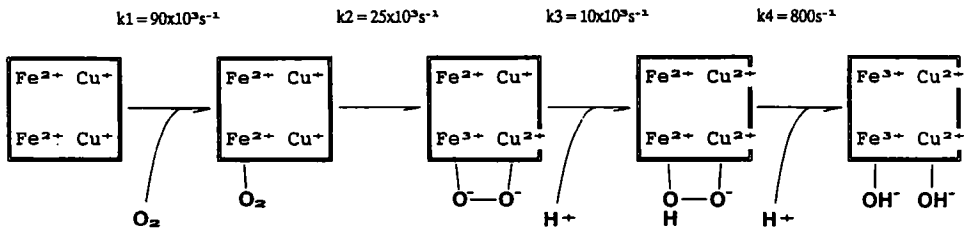

Fig. 7. Model for the sequence of reactions between fully reduced cytochrome oxidase and O_2.

of a comparison with low-temperature results [22], this has been suggested [18] to lead to the formation of an oxyferryl cytochrome a_3-Cu_B (II) species. Following Chan et al. [19], we have, however, assumed that this is preceded by an intermediate in which cytochrome a_3 is reduced and the peroxide is singly protonated. This would agree with the clear dependence of the rate of this step on the uptake of one proton. The assignments are also supported by the absorbance increase at 605 nm, since according to Wikström [23] the peroxy intermediate has a higher absorbance at this wavelength than the ferryl ion species. In addition, the very small temperature dependence of the rate makes it unlikely that the O–O bond is broken in this step.

Orii [9] assumes that the third phase represents a conformational change of the peroxy species without any electron transfer from Cu_A. His main reason for this appears to be that he doubts that electron transfer from Cu_A can be more rapid than that from cytochrome *a*. Rapid electron transfer from Cu_A to the binuclear site, and slow transfer from cytochrme *a*, has, however, been clearly demonstrated in anaerobic experiments with the mixed-valence oxidase [3,24]. The transfer of an electron from Cu_A in the third reaction phase is also supported by resonance Raman spectra, which show that an intermediate with cytochrome a_3 in the Fe^{2+}–O_2 or $Fe^{4+}=O$ state and cytochrome *a* in the Fe^{2+} state is formed in 450 μs [11].

In the final step the fourth electron is transferred to the binuclear site from cytochrome *a*, and the O–O bond is broken. This would agree with the somewhat greater temperature dependence of the rate of this step. Again, the pH dependence of the rate indicates that only one proton is involved, so that OH^- should be bound to both cytochrome a_3 and Cu_B in the final species formed. This agrees with the structure for pulsed oxidase suggested by Chan et al. [19], who assume that the final two protons needed to form two molecules of water originate from protonated amino acid residues in the catalytic site.

We cannot say if cytochrome *a* transfers its electron directly to the binuclear site, or if this occurs via Cu_A. A comparison with results for the mixed-valence enzyme [3] supports the latter alternative, however, since it was concluded that the rate constant for electron transfer from cytochrome *a* to Cu_A was close to 800 s^{-1}.

Starting from the fully oxidized, pulsed oxidase, the intramolecular electron transfer following the reduction of cytochrome *a* and Cu_A is slow and thought to provide the main limit on k_{cat}, the catalytic constant [25,26]. In the reoxidation of the fully reduced enzyme, however, even the slowest step has a rate constant which is much larger than k_{cat}. This cannot be ascribed to the higher driving force in the presence of oxygen [25], because almost identical rate constants for the oxidation of Cu_A and cytochrome *a* are found anaerobically in the mixed-valence oxidase [3]. Instead, it can be related to our earlier suggestion [3] that there is a high activation energy for the internal electron transfer in the initial electron input state, but that the reduction of the primary electron acceptors leads to a conformational transition, which drastically reduces this barrier (electron gating).

The rate of electron transfer in the slowest step at the highest pH used is still higher than k_{cat}. Thus, the pH dependence of k_{cat} [27] cannot be related to the consumption of protons in dioxygen reduction.

It may seem surprising that the anaerobic electron transfer rates from Cu_A and cytochrome *a* in the mixed-valence oxidase are as high as those in the reoxidation of the fully reduced enzyme with O_2, since the driving force is at least 0.5 V higher in the latter case [28]. This can probably be explained by an increase in the reorganization energy, λ, because of the oxygen chemistry that must go on in the aerobic reaction. According to Marcus theory [7] the activation energy for electron transfer can be determined by a tradeoff between λ and the driving force, $-\Delta G^0$, and it becomes zero when $-\Delta G^0 = \lambda$. The very small temperature dependence found here, as well as in the anaerobic reaction in the mixed-valence state [3], for the internal electron-transfer steps suggests indeed that there is a close balance between the driving force and the reorganization energy. The negative entropies of activation calculated from transition-state theory also indicate that the rates of electron transfer are largely limited by structural rearrangements.

Our results with the mixed-valence enzyme agree essentially with those of Hill and Greenwood [12], who studied the reaction between this enzyme form and O_2 at a single pH and temperature, even if we do find a somewhat lower rate constant for the third phase. We suggest a different structure for the second intermediate, however, as illustrated in Fig. 8. The reason for this is a comparison with model systems [29], which shows that oxidation of a reduced binuclear model complex with O_2 immediately leads to the formation of a μ-peroxy bridge. Thus, the structure proposed earlier [12], with the peroxide coordinated to oxidized cytochrome a_3 but not to Cu_B^{2+}, does not appear to be chemically reasonable.

If our structural assignment (Fig. 8) is correct, it is remarkable that the peroxide intermediate is formed considerably more rapidly ($2.5 \cdot 10^4$ s^{-1}) when starting from the fully reduced enzyme compared to the mixed-valence state (6000 s^{-1}). Thus, it appears that the structure of the binuclear site depends on the redox state of the other two sites. It has earlier been shown that there are both spectral [30] and redox interactions [31] between the binuclear site and the primary electron acceptors, cytochrome *a* and Cu_A. In addition, experiments with cyanide [32,33] have shown that the cyto-

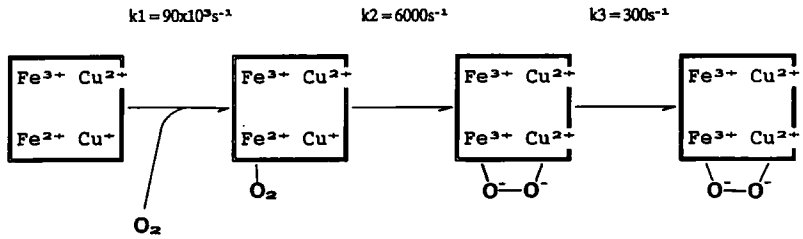

Fig. 8. Model for the sequence of reactions between mixed-valence cytochrome oxidase and O_2.

chrome a_3-Cu_B site has a more 'open' structure, when cytochrome a and Cu_A are reduced compared to the state when they are oxidized.

The first two reactions in Fig. 8 are not expected to involve H^+, which agrees with the lack of a pH dependence (Fig. 6). The third phase cannot involve any electron transfer and must consequently represent some structural change, in agreement with the somewhat larger temperature dependence. A comparison with a reaction cycle proposed earlier [3,4] for cytochrome oxidase as a proton pump suggests that this reaction may be the return of the enzyme from the electron-output (E_2) to the electron-input state (E_1). One would, however, expect this to be inhibited by H^+ [34], which is not in agreement with the results in Fig. 6. On the other hand, recent findings [35] suggest that protonated E_2 can also return to E_1, which leads to an intrinsic uncoupling.

The lack of temperature dependence of all three reaction phases indicates that the reorganization energy [7] is always closely balanced by the driving force, as already discussed for the reaction of the fully reduced enzyme with O_2. The large negative entropies of activation also indicate that the rates are limited by structural changes.

Acknowledgements

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