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THE EFFECT OF pH AND IONIC STRENGTH ON THE PRE-STEADY-STATE REACTION OF CYTOCHROME *c* AND CYTOCHROME aa_3

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(1) In the pH range between 5.0 and 8.0, the rate constants for the reaction of ferrocytochrome *c* with both the high- and low-affinity sites on cytochrome aa_3 increase by a factor of approx. 2 per pH unit. (2) The pre-steady-state reaction between ferrocytochrome *c* and cytochrome aa_3 did not cause a change in the pH of an unbuffered medium. Furthermore, it was found that this reaction and the steady-state reaction are equally fast in H_2O and ^2H_2O. From these results it was concluded that no protons are directly involved in a rate-determining reaction step. (3) Arrhenius plots show that the reaction between ferrocytochrome *c* and cytochrome aa_3 requires a higher enthalpy of activation at temperatures below 20°C (15–16 kcal/mol) as compared to that at higher temperature (9 kcal/mol). We found no effect of ionic strength on the activation enthalpy of the pre-steady-state reaction, nor on that of the steady-state reaction. This suggests that ionic strength does not change the character of these reactions, but merely affects the electrostatic interaction between both cytochromes.

Introduction

The effects of pH and salt concentrations of the medium on the steady-state activity of cytochrome aa_3 have received much experimental attention from several investigators [1–5]. In a recent paper from our group [5], we have shown that the pH affects both the affinity between cytochrome *c* and cytochrome aa_3 and the overall rate-limiting step, which is related to the intramolecular electron transfer from cytochrome *a* to a_3 within the oxidase molecule [6]. In this paper, the effect of pH on the pre-steady-state reaction between ferrocytochrome *c* and cytochrome aa_3 will be presented for both the high and low ionic strength regions. It has been demonstrated that at low ionic strength [7–10], a 1 : 1 complex between cytochrome *c* and cytochrome aa_3 is a stable, though less active, reaction partner of ferrocytochrome *c*. The large difference in reactivity has been explained as the partial shielding of the cytochrome *c*-binding region on cytochrome aa_3 [10].

A more direct approach to appreciate the role of protons in the reaction mechanism is the measurement of proton release or uptake during the time course of the reaction. The isotopic substitution of ^1H_2O by ^2H_2O can provide similar information and has been reported to inhibit the steady-state reaction by about 40–60% [11–14].

The decrease in the pre-steady-state activity at higher ionic strength has been explained as a primary salt effect, i.e., the shielding of opposite electrical charges on the two reactants by different ions. An alternative explanation might be that the decrease is caused by a shift to a slower, rate-controlling reaction step at higher ionic strength. For this reason, the temperature dependences of both steady- and pre-steady-state reactions were determined at various ionic strengths. The high values for the enthalpy of activation which were found are a strong indication

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that these reactions are not diffusion limited [15,16].

In the interpretation of more complicated mechanisms, such as that from the reduction level of the various cytochromes during oxidative phosphorylation, an estimation is needed for the rate at which ferrocytochrome *c* and cytochrome aa_3 associate. The values most often cited [6,17–19] are only applicable to a very limited range of experimental conditions. The experimental values for the rate constant of the pre-steady-state reaction between both cytochromes presented in this study allow a more accurate estimation of this rate over a wide range of ionic strength, pH and temperature. Furthermore, the effect of these parameters on the rate of reaction between cytochrome *c* and cytochrome aa_3 may contribute to the elucidation of the in vivo regulation of mitochondrial respiration.

Materials and Methods

Cytochrome aa_3 was isolated from fresh beef heart by a modification of the method of Fowler et al. [20–22]. Cytochrome *c* was isolated from horse heart [23], reduced with excess ascorbate and after gel filtration [24] stored in liquid nitrogen. Absorbance coefficients (reduced minus oxidized) used were 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm for cytochrome aa_3 [25] and 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm for cytochrome *c* [26]. Steady-state activity of cytochrome aa_3 was determined according to the method of Smith and Conrad [27].

The time course of various reactions was studied using a Durrum stopped-flow apparatus (2.0 cm optical pathlength of the reaction chamber) equipped with a Lauda-MGW K2R-D thermostat keeping the temperature constant to within 0.1 °C.

Data handling and evaluation of reaction rates have been described previously [9]. The applied non-linear least-squares curve fit was effected using Marquardt's algorithm [28,29] in selecting the gradient of parameter adjustment. Since the convergence of the method may depend on the quality of the initial guess [30], the iteration is initiated by values obtained from a linear regression applied to the logarithm of the difference between experimental values and (estimated) final level(s). The program is written in Fortran IV; some of the subroutines used in this program are described and listed in Ref. 31.

In experiments in which H_2O was replaced by ^2H_2O, the 100 mM potassium phosphate buffer was prepared by diluting a stock solution (in H_2O) of 2 M potassium phosphate, pH 7.0, in H_2O and in 99.75% ^2H_2O (Uvasol, Merck), respectively.

Changes in proton concentration accompanying the reaction between ferrocytochrome *c* and cytochrome aa_3 in an unbuffered medium were followed spectrophotometrically in the presence of a pH indicator (30 μM phenol red, pK_a 7.9). Both cytochrome *c* and cytochrome aa_3 were depleted of buffering ions by the method of Penefsky [32]. Changes in pH were monitored at 541 nm, a wavelength isosbestical to redox changes in both cytochromes. Solutions were depleted of CO_2 with a vacuum pump and the pH was adjusted with 1 M KOH or HCl, under a flow of CO_2-free air. Proton yields were corrected for buffer capacity in the presence of both cytochromes by measuring the shift in final absorbance at 541 nm, caused by the addition of known amounts of oxalic acid. Maximal pH changes in the total time course of the reaction were less than 0.1 unit as measured in experiments in which pH and optical changes were detected simultaneously in a stirred, 10 ml cell with an optical pathlength of 2 cm; spectral changes were recorded with a Cary 17R spectrophotometer and the pH was measured with a Philips PW9408 pH/mV meter using an Ingold micro pH-electrode. For pH-stat experiments, the very accurate equipment described in Ref. 33 was used.

Ionic strength of potassium phosphate buffers was calculated as described in Ref. 5 in an iterative procedure. The Davies approximation was used for ionic strength effects on the pK_a values [34]:

$$pK_a = pK_a^0 + B \cdot (Z_{HA}^2 - Z_A^2) \cdot \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.2I \right)$$

where Z_{HA} and Z_A are the charge numbers of the protonated and unprotonated forms of the acid, respectively, pK_a^0 is the pK_a at zero ionic strength and B has the value of 0.509 at 25 °C. Corrections for temperature effects on pK_a values were taken from Ref. 35.

Results

The time course of the aerobic reaction of ferrocytochrome *c* and cytochrome aa_3 in an unbuffered

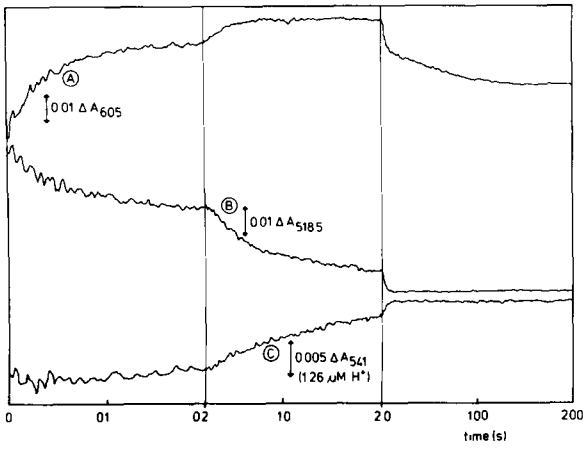


Fig. 1. Time course of the aerobic reaction between ferrocytochrome *c* and cytochrome aa_3 in the presence of phenol red. Conditions: 5 μM ferrocytochrome *c*, 1 μM cytochrome aa_3, 30 μM phenol red, 200 mM KCl, 1% Tween 20, pH 7.5, 10°C. At the indicated wavelengths are monitored: A, cytochrome aa_3; B, cytochrome *c* and C, phenol red, the absorbance change being related to changes in proton concentration by calibration with oxalic acid.

medium and in the presence of the pH indicator phenol red is presented in Fig. 1. Trace A shows the absorbance changes at 605 nm, a wavelength where the redox changes in cytochrome aa_3 are detected. Three phases can be easily recognized: pre-steady state, steady state and post-steady state. Initially, the oxidase is partially reduced by electron transfer from ferrocytochrome *c*. In this experiment, the applied ionic strength is rather high and no distinction between the reactions of ferrocytochrome *c* with high- or low-affinity sites on cytochrome aa_3 is found [9,10].

After the pre-steady-state reduction of the oxidase, a constant reduction level of heme *a* is reached during the steady-state electron flow through cytochrome aa_3. Finally, a very slow decrease in absorbance occurs in the post-steady state, which is ascribed to the reoxidation of the partially reduced cytochrome aa_3 by molecular oxygen. After about 10–25 min the initial absorption level of the fully oxidized enzyme is reached again.

The redox changes in cytochrome *c* are monitored at 518.5 nm (trace B), a wavelength where the total absorbance change depends neither on the dissociation state of the pH indicator nor on the redox state of cytochrome aa_3. The enzyme-catalyzed oxidation of ferrocytochrome *c* by molecular oxygen follows the well known exponential time course in the steady-state phase.

Trace C shows the absorbance changes at 541 nm, where only pH changes contribute to the total absorbance changes. It is clear that both the pre-steady- and the post-steady-state phases are not accompanied by detectable pH changes of the unbuffered medium. In the steady state, however, a change in proton concentration is observed which is concomitant with and in almost 1 : 1 stoichiometry to the steady-state oxidation of ferrocytochrome *c*. This can also be deduced from the steady-state equation:

$$4 \text{ cytochrome } c^{2+} + 4\,H^+ + O_2 \xrightarrow{\text{cytochrome } aa_3} 4 \text{ cytochrome } c^{3+} + 2\,H_2O$$

The 1 : 1 electron/proton ratio (i.e., one ferrocytochrome *c* oxidized per proton consumed) is also found during oxidation of ferrocytochrome *c* (10–50 μM) in the presence of catalytic concentrations of cytochrome aa_3 (10–40 nM). Similar experiments, with simultaneous detection of the optical changes at 550 nm and of the proton concentration using a micro pH-electrode (or accompanied by a very accurate pH-stat equipment), confirmed this 1 : 1 ratio in the pH range from 6.7 to 7.8 (not shown).

The absence of any absorbance changes at 541 nm in the pre- and post-steady-state phases of the reaction is evidently not due to the insensitivity of the assay method; addition of 120 nM oxalic acid caused a clearly detectable decrease of the final absorbance level of 0.001 absorbance units (30 μM phenol red, pH 7.5, 200 mM KCl, 1% Tween 20, 1 μM cytochrome aa_3, 5 μM cytochrome *c*).

In the absence of phenol red, no absorbance changes occur at 541.0 nm (0.5 nm optical bandwidth), whereas the absorbance changes at 605 and 518.5 nm are identical to those shown in trace A and B, respectively. This demonstrates that the presence of the pH indicator does not interfere with the electron-transfer reactions, as could be confirmed for the steady-state reaction (10–40 nM cytochrome aa_3, 5–25 μM ferrocytochrome *c*) using a Cary 17R instead of a stopped-flow spectrophotometer.

As can be seen in Fig. 2, isotopic substitution of up to 90% of H_2O by ^2H_2O had no effect on any of the three reaction phases. Moreover, when the steady-

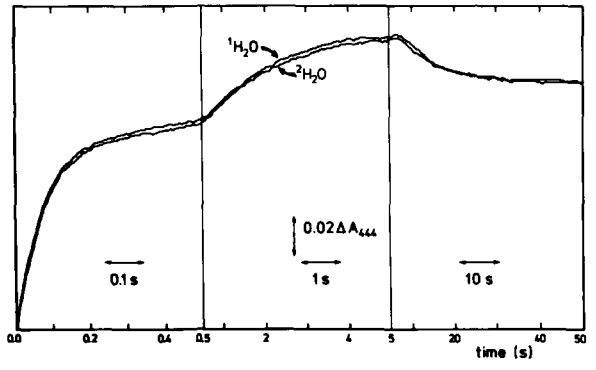


Fig. 2. Time course of the aerobic reaction between ferrocytochrome *c* and cytochrome aa_3 in H_2O and in ^2H_2O (90%). Conditions: 7.5 μM ferrocytochrome *c*, 0.8 μM cytochrome aa_3, 100 mM potassium phosphate, pH 7.0, 1% Tween 20, 10°C.

state activity of cytochrome aa_3 was determined according to the method of Smith and Conrad [27], no effect of ^2H_2O on the catalytic activity of the enzyme was observed. The complete absence of effects of ^2H_2O is in contrast with earlier observations of other workers [11–14].

The role of the protons in the pre-steady-state reaction of ferrocytochrome *c* and cytochrome aa_3 was also studied indirectly as the effect of pH on the association rate. Two phases can be discerned in this pre-steady-state reaction [9,10,36]: the initial reaction of cytochrome *c* with the high-affinity site and a subsequent reaction via the low-affinity site. At low ionic strength, the reaction with the high-affinity site is too fast to be measured with the stopped-flow technique and only the reaction of the initially formed 1 : 1 cytochrome *c*-cytochrome aa_3 complex with the excess (if present) ferrocytochrome *c* will be observed [9]. Fig. 3A (left-hand part) shows the second-order rate constant of this (low-affinity site) reaction, determined from a cytochrome *c* range of 3.5–10 μM, as a function of both ionic strength and pH. As

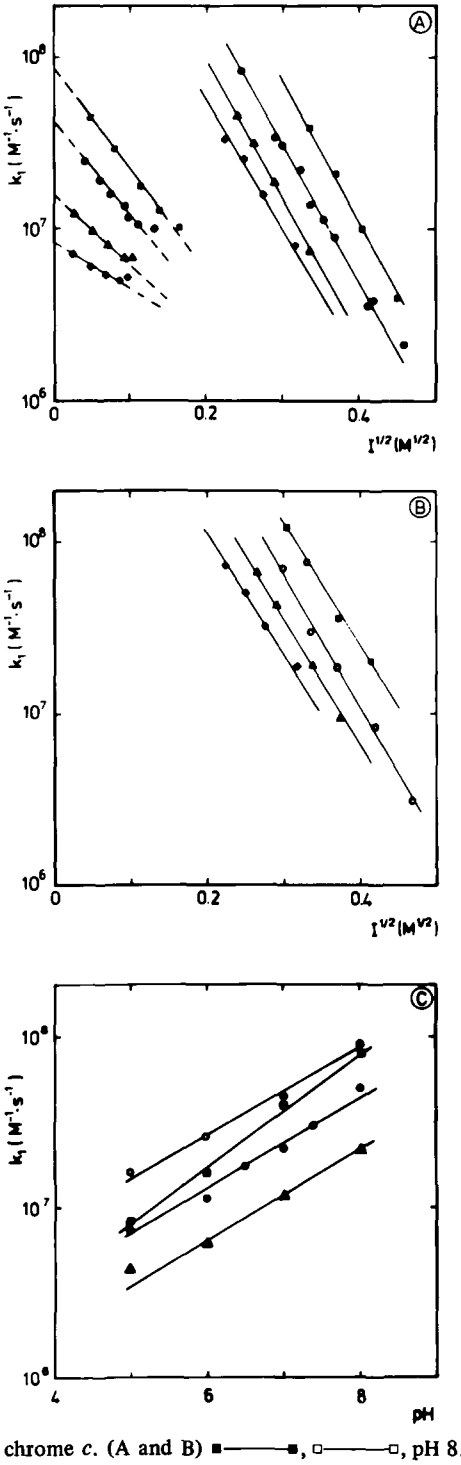


Fig. 3. Effect of ionic strength on the rate constant of the pre-steady-state reaction between cytochrome *c* and cytochrome aa_3 at various pH values. Conditions: 1–125 mM potassium phosphate, 1% Tween 20, 10°C. Solid symbols: 3.5–10 μM ferrocytochrome *c*, 0.8 μM cytochrome aa_3. Open symbols: 3–8 μM cytochrome aa_3, 0.8 μM ferrocytochrome *c*. (A and B) ■——■, □——□, pH 8.0; ●——●, ○——○, pH 7.0; ▲——▲, △——△, pH 6.0; ◆——◆, ◇——◇, pH 5.0. (C) ●——●, ○——○, I = 100 mM; ▲——▲, I = 10 mM, ■——■, I = 1 mM.

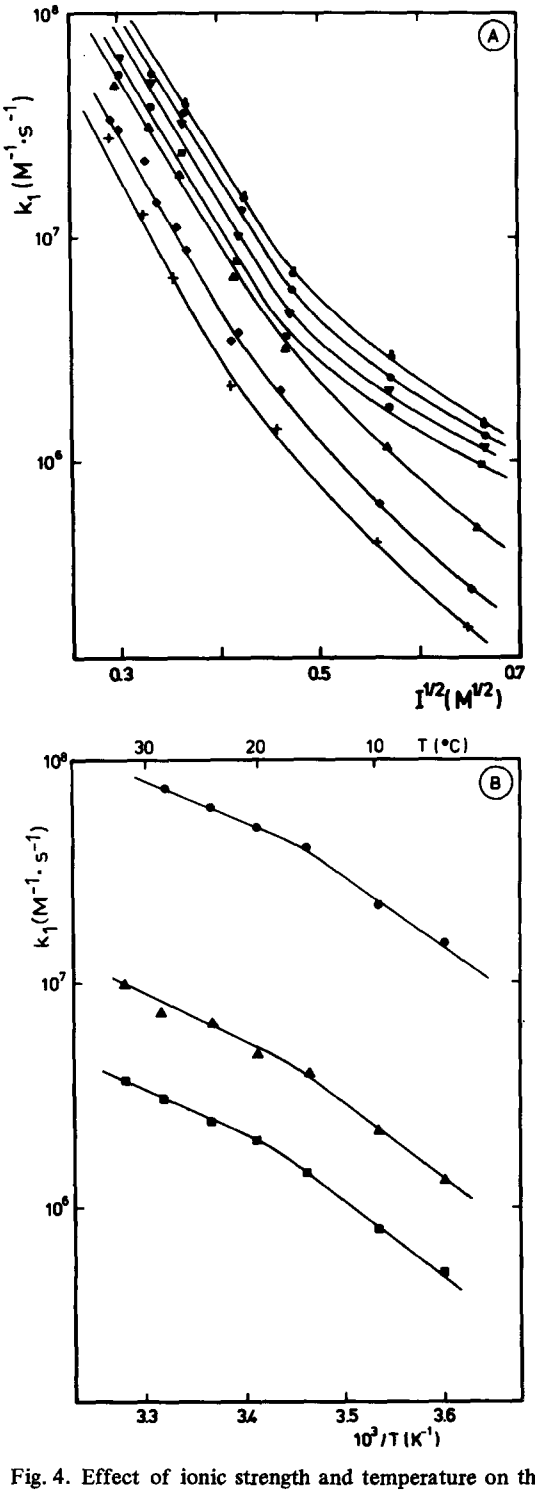


Fig. 4. Effect of ionic strength and temperature on the pre-steady-state reaction of ferrocytochrome *c* and cytochrome aa_3. Conditions: 7.5 μM ferrocytochrome *c*, 0.8 μM cytochrome aa_3, 50–240 mM potassium phosphate, 1% Tween 20, pH 7.0. (A) ♦——♦, 32°C; ●——●, 28°C; ▼——▼, 25°C; ■——■, 20°C; ▲——▲, 15°C; ◆——◆, 10°C; +——+, 5°C. (B) ●——●, I = 100 mM; ▲——▲, I = 200 mM; ■——■, I = 300 mM.

has been shown previously [10], the rate of this reaction decreases continuously with increasing ionic strength. It is clear that both the rate and steepness of the lines in the log k_1 vs. $\sqrt{I}$ plot increase with increasing pH. The slopes, which are a measure of the electrostatic interaction involved, shift from −2.8 to −6.1 when the pH increases from 5 to 8. In the ionic strength region from approx. 30 to 50 mM, the contributions of the high- and low-affinity site reactions could not be separated reproducibly into two exponentially decreasing absorbance changes, the more so as the effect of a consecutive reoxidation of cytochrome aa_3 was not negligible under these conditions.

The reaction of ferrocytochrome *c* with the high-affinity site is also slowed down by increasing the ionic strength (cf. Fig. 3A, right-hand part). The second-order rate constant of this reaction has been determined for $I > 50$ mM at various pH values, both in the case when cytochrome *c* is present in excess (Fig. 3A) and when cytochrome aa_3 is present in excess (Fig. 3B). Again, both the rate and the steepness of the line increase with increasing pH, although the latter effect is much less profound (the slopes shift from −7.5 at pH 5 to −8.8 at pH 8) than that for the low-affinity site reaction in the low ionic strength region. Note, moreover, that the ratio k_1([cytochrome aa_3] > [cytochrome *c*])/k_1([cytochrome *c*] > [cytochrome aa_3]) is about 2 and does not depend on the pH. This is shown more explicitly in Fig. 3C; the open and solid circles refer to the second-order rate constants, interpolated to I = 100 mM, for [cytochrome aa_3] > [cytochrome *c*] and for [cytochrome *c*] > [cytochrome aa_3], respectively. The pH dependences of the association rates at both high and low ionic strength are in general accordance with the results obtained for the Michaelis constant K_m of the steady-state reaction [5].

The ionic strength dependence of the reaction with the high-affinity site is presented in Fig. 4A for the temperature range from 5 to 32°C. Up to ionic strength values of 200 mM, the second-order rate

constants give a series of almost parallel lines in this Brønsted-type plot with slopes that shift from −7.9 at 32°C to −8.2 at 5°C. At higher ionic strength the data show a clear deviation from linearity, as may be expected for this type of plot on theoretical grounds [37,38]. Since the pK_a values of the applied salt (potassium phosphate) are also temperature dependent, interpolation from the results presented in Fig. 4A was applied to obtain the temperature dependence of the reaction rate constants at fixed ionic strength. This is shown in the Arrhenius plots of Fig. 4B for I = 100, 200 and 300 mM. At these three ionic strength values, the plots show a slight bend at about 20°C. Below this temperature the enthalpy of activation, $\Delta H^{\ddagger}$, is approx. 15.5 kcal/mol, above this temperature about 8 kcal/mol.

The fact that the activation enthalpy remains constant at various ionic strengths implies that the difference in reaction rate is due to a smaller increase in activation entropy upon association at increasing ionic strength (at 25°C, 27 cal/mol per K at I = 100 mM and 24 cal/mol per K at 300 mM).

Fig. 5 shows, for comparison, the temperature dependence of the steady-state activity of cytochrome aa_3 at various ionic strengths. Again, a slight bend is observed at about 20°C, as is often observed for membrane-bound enzymes [39–41]. Furthermore, the activation enthalpy for both the low- and high-temperature regions (17.8 and 6.6 kcal/mol, respectively) is almost independent of ionic strength.

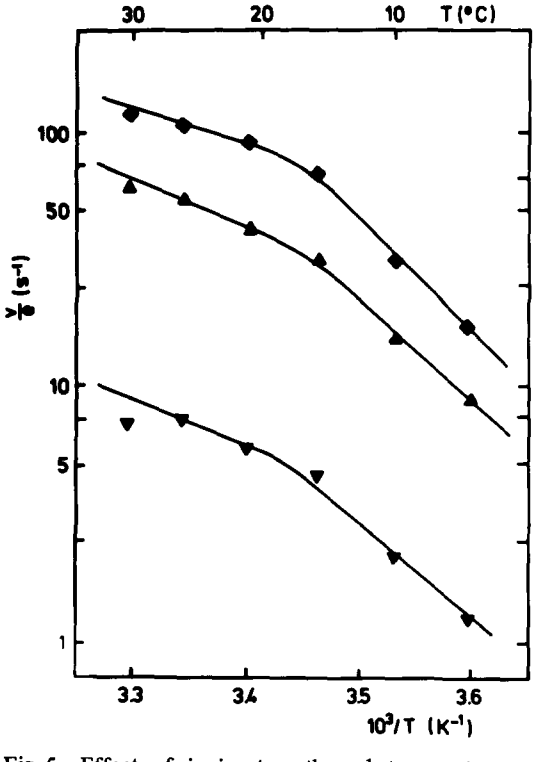
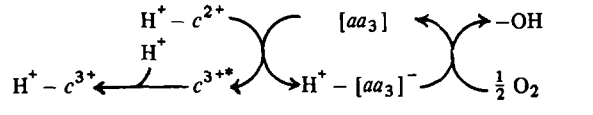


Fig. 5. Effect of ionic strength and temperature on the steady-state activity of cytochrome aa_3. Conditions: 7.5 μM ferrocytochrome *c*, 8 nM cytochrome aa_3, 50–250 mM potassium phosphate, 1% Tween 20, pH 7.0. ♦——♦, I = 88 mM; ▲——▲, I = 176 mM; ▼——▼, I = 440 mM.

Discussion

It is obvious that the sensitivity of the pH-change detection is very important for experiments in which proton uptake or release is measured. From the experiments in the absence of buffer and in the presence of a pH indicator shown in Fig. 1, it is clear that the sensitivity of our assay suffices to monitor small pH changes; the absorbance increase at 541 nm in the first stage of the steady-state reaction can be clearly detected, nevertheless the amount of cytochrome *c* oxidized in that stage is only 10–20% of the amount of cytochrome *c* involved in the pre-steady-state reaction. Since it is likely that proton exchange between aqueous medium and cytochromes or pH indicator is fast, the absence of pH changes in the pre- as well as in the post-steady-state points to the fact that no redox-coupled uptake or release of protons occurs apart from the one proton per electron observed in the steady state. The absence of a pH change is remarkable especially in the post-steady-state reoxidation of cytochrome aa_3.

A possible explanation for these observations is the hypothesis that a proton release or uptake, induced by the change in redox state of a cytochrome, is accompanied by a compensating pK_a shift and/or conformational change. Such a mechanism is, e.g.:



For simplicity only the reduction of $\frac{1}{2}O_2$ is considered; the complete reduction and protonation of O_2 to $2H_2O$ may proceed via an analogous mechanism. According to this scheme, the transfer of an

electron from ferrocytochrome *c* to oxygen is accompanied by a stoichiometric proton transfer. After the intramolecular transfer of a proton, the reduction of molecular oxygen leads to the formation of a protonated oxygen intermediate, similar to the formation of oxygenated cytochrome aa_3 from the fully reduced enzyme [42,43]. This intermediate (-OH) may be the cytochrome a_3 hydroxyl ligand as reported by Lanne et al. [44] which might play a rate-determining role in the steady-state activity of cytochrome aa_3 [5].

After the reaction with a second ferrocytochrome *c* molecule and the co-transfer of a proton, water will be formed from the -OH ligand. This final step may also be interpreted as a shift in pK_a, induced by a redox change of the high-potential heme, as has been reported by Van Gelder et al. [45].

The proton uptake in the steady-state reaction has to take place in the relatively slow transition [46] of the unstable conformation of cytochrome c^{3+*} (possibly a more compact cytochrome *c* conformation [47,48]) into cytochrome c^{3+} after dissociation from the oxidase [49]. The absence of an effect of ^2H_2O on the reaction is in accordance with the results of Kihara et al. [50] and with the preceding hypothesis, since there is no direct 'kinetic' role for protons in any rate-determining step. It should be borne in mind, however, that a rate-determining step in which protons are involved is not necessarily slowed down by substituting ^1H by ^2H [51].

The inhibitory effect of ^2H_2O on the steady-state activity of cytochrome aa_3 reported by several authors [11–14] can be explained only partly by the involvement of another rate-limiting step, i.e., proton translocation by the oxidase in intact mitochondria or in submitochondrial particles [52]. However, Tyler and Estabrook [13] found also inhibition with isolated cytochrome aa_3. The difference between their results and ours must be attributed to the different experimental conditions (cf. Ref. 57), such as correction for the ^2H_2O-induced pH shift, and the different assay method: oxygen-uptake measurement in the presence of ascorbate as compared to the spectrophotometric assay of Smith and Conrad [27]. It should be mentioned that the first method is sensitive to an erroneous interpretation of effects [54] caused by a shift to another rate-determining step [55,56].

There may be theoretical objections against a quantitative interpretation of the value of the slope in a log *k* vs. $\sqrt{I}$ plot being simply the product of the electrical charges of the two reactants [15,16,38]. Qualitatively, however, the increasing steepness of the lines in the low ionic strength region in Fig. 3A is a clear indication that the contribution of the electrostatic interaction between ferrocytochrome *c* and the cytochrome *c*-aa_3 complex increases with pH. The increased intermolecular attraction at higher pH can be explained by an increased number of negative charges on the cytochrome *c*-aa_3 complex at lower proton concentration.

In the high ionic strength region, a similar, though less explicit, pH dependence of the slope is observed. Apparently, the negative charge on the high-affinity site is less sensitive to pH changes than the low-affinity site. It is obvious that cytochrome *c* will occupy preferentially the most negative part of its binding region on cytochrome aa_3. The cytochrome *c*-aa_3 complex thus formed will have a less negatively charged region to act as a low-affinity site for cytochrome *c* [10].

The relatively high value of the enthalpy of activation (approx. 15.5 kcal/mol) indicates that, in spite of the high value of the second-order rate constant, the pre-steady-state reaction is not diffusion limited, since a much lower $\Delta H^{\ddagger}$ can be expected in that case [15]. Our results show that the ionic strength hardly affects the activation enthalpy of both steady- and pre-steady-state reactions. This observation is in line with the conclusion from Amis [57] that a change in the electrostatic interaction between two reactants does not cause an appreciable change in the activation enthalpy. Evidently, no shift to another rate-controlling step occurs upon increase of ionic strength. The effect of ionic strength on the reaction rate is therefore limited to a change in the activation entropy, $\Delta S^{\ddagger}$. This is consistent with the idea that the gain of configurational arrangements of surrounding molecules (anions, cations, water) upon combination of the two electrically charged cytochromes is important in the realization of their rapid association. At higher ionic strength the electrostatic-influence spheres of the charges on both cytochromes will be diminished [10] and the entropy gain will accordingly decrease.

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