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THE EFFECT OF pH ON THE OXYGEN KINETICS OF CYTOCHROME *c* OXIDASE PROTEOLIPOSOMES

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Summary

The effect of pH on the oxygen kinetics of cytochrome *c* oxidase incorporated into phospholipid vesicles is studied. The pH profiles of the oxygen kinetics of energized and deenergized oxidase vesicles are similar. An effect of pH on the slope of the reciprocal plot of rate against oxygen concentration is observed, and this may indicate that protons are involved in the rate limiting step of the reaction between oxygen and reduced oxidase. In contrast to the pH dependence of the oxygen kinetics, the binding of CO to the oxidase is not pH dependent.

It is of general interest to obtain information about the pH profiles of the kinetics of an enzyme. The information is of particular interest if protons can be considered as being a substrate for the enzyme. Cytochrome *c* oxidase catalyzes the reaction

$$4\ \text{Cyt}\ c\ (Fe^{2+}) + O_2 + nH^+ \rightarrow 4\ \text{Cyt}\ c\ (Fe^{3+}) + 2H_2O + (n-4)H^+ \qquad (1)$$

and as indicated by the scheme several protons interact with intermediates of the reaction. It is necessary to consider not only the four protons needed for the actual reduction of O_2, but also any extra protons that might be involved in the postulated proton pumping activity of the enzyme [1—3]. Information about the reaction mechanism of cytochrome oxidase has been obtained from a number of kinetic studies including recent investigations on the steady state oxygen kinetics of cytochrome *c* oxidase [4—8].

The present communication describes the effect of pH on the oxygen kinetics of the oxidase incorporated into liposomes and discusses the possible interaction of intermediates of the reaction with protons.

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Abbreviation: FCCP, trifluoromethoxycarbonyl cyanide phenylhydrazone.

Beef heart cytochrome *c* oxidase was purified according to the method described by van Buuren [9]. Proteoliposomes were prepared according to the procedure (alternative b) described by Hansen et al. [7]. Cytochrome *c* oxidase (0.026 mg protein/mg phospholipid) was added to a mixture of phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin (4:4:2, v/v). Oxidase vesicles prepared by this method have a respiratory control ratio of about 2.5 calculated from the oxygen consumption rate in the presence of ascorbate and cytochrome *c*.

Phospholipids, were from Lipid Products Inc. Nutfield Nurseries, Surrey, U.K. Cytochrome *c* (type VI) was from Sigma Chemical Co.

The oxygen kinetics of the oxidase reaction were measured using the open system as in previous studies [4—8]. A detailed account of this technique is given in [10].

The CO binding was measured in a Cary 118 C spectrophotometer using the method described in Ref. 11. The concentration of oxidase was calculated using $\epsilon_{605-630}$ (reduced-oxidized) = 27.000 $l \cdot mol^{-1} \cdot cm^{-1}$. The concentration of the CO complex was calculated from the peak at 430 nm after small aliquots of buffer saturated with 5% CO in N_2 were added to the dithionite reduced oxidase. The concentration of free CO was calculated by subtracting this value from the total concentration of CO.

Fig. 1 shows the oxygen kinetics obtained with 'energized' oxidase vesicles at six different pH values ranging from 5.9 to 8.4. The experiments are performed in 67 mM potassium phosphate in the presence of 22 mM ascorbate and 45 μM cytochrome *c*. In agreement with previous experiments on

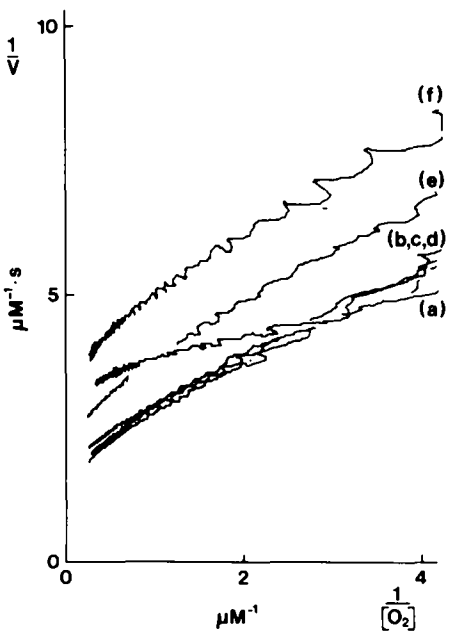

Fig. 1. Oxygen kinetics of energized cytochrome aa_3 proteoliposomes at various pH values. Reciprocal plots of rate against oxygen concentration. Additions: 30 nM cytochrome aa_3 proteoliposomes, 45 μM cytochrome *c*, 22 mM ascorbate, 67 mM potassium phosphate. pH: a, 5.9; b, 6.4; c, 6.9; d, 7.4; e, 7.9 and f, 8.4.

oxidase vesicles [7] and outer membrane stripped mitochondria [12] non-linear reciprocal plots of rate against oxygen concentration are obtained in the absence of uncoupling reagents. The pH of the medium seems to affect both the slope and the intercept of these plots.

The effect of pH on the oxygen kinetics of the oxidase vesicles in the deenergized state is shown in Fig. 2A. Visicles are added at a 2.5 times lower

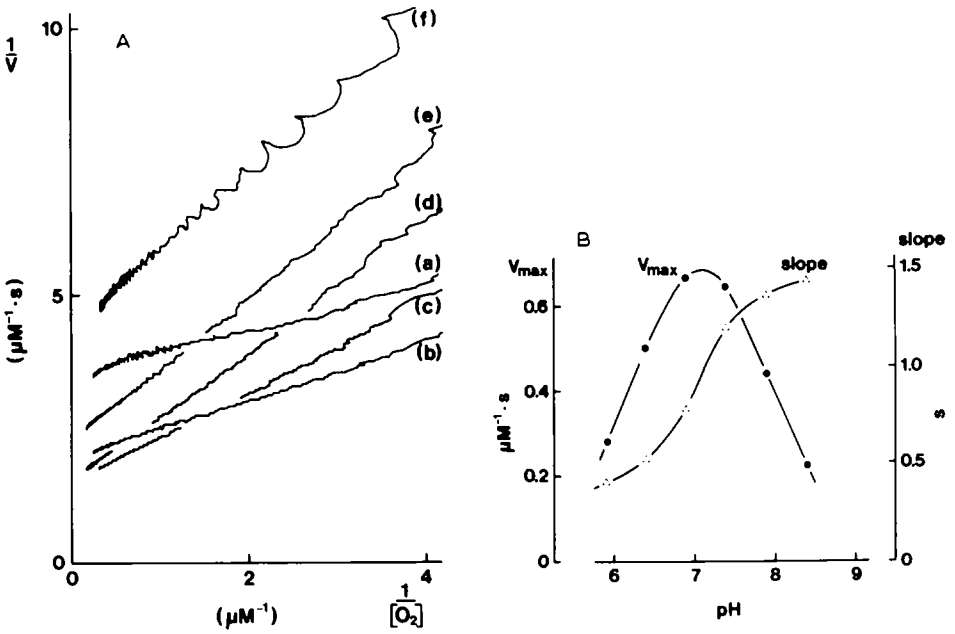

Fig. 2. (A) Oxygen kinetics of de-energized cytochrome aa_3 proteoliposomes at various pH values. Reciprocal plots of rate against oxygen concentration. Additions: 12 nM cytochrome aa_3 proteoliposomes, 45 μM cytochrome *c*, 22 mM ascorbate, 3.3 μM FCCP, 0.66 μg/ml valinocycin, 67 mM potassium phosphate. pH: a, 5.9; b, 6.4; c, 6.9; d, 7.4; e, 7.9 and f, 8.4. (B) Secondary plots of slope and maximal oxygen uptake rate for the de-energized proteoliposomes as a function of pH. The values are obtained from the Lineweaver-Burk plots presented in Fig. 2A.

concentration than in Fig. 1 and the respiration is uncoupled by addition of 0.66 μg/ml valinomycin plus 3.3 μM FCCP. Linear plots are obtained at each value of pH. Secondary plots are shown in Fig. 2B. The maximal respiration rate (calculated from the intercept) and the slope of the Lineweaver-Burk plots is shown as a function of pH. A similar pH effect on the slope is obtained with solubilized purified oxidase or submitochondrial particles (experiments not shown). In accordance with previous observations [13] the pH optimum for maximal respiration of oxidase proteoliposomes is somewhat higher than that obtained with purified oxidase [14,15]. Cytochrome oxidase incorporated into liposomes is an unusually stable oxidase preparation [3] and results obtained with purified solubilized oxidase at extreme pH values tend to be less reproducible.

The plot of slope versus pH presented in Fig. 2B suggests that pH has an effect on the rate at which the free enzyme (reduced oxidase) combines with its substrate (oxygen) to form products [16]. Much experimental evidence suggests that this reaction is practically irreversible at room tem-

perature (see e.g. Refs. 6 and 17) despite of the fact that a reversible binding of oxygen has been demonstrated at low temperatures [18]. This property of the reaction implies that the second order constant, k_{on}, for the oxidation of cytochrome oxidase by oxygen is inverse proportional to the slope, α, of the Lineweaver-Burk plot of the oxygen kinetics. It is: $\alpha = 1/e\ k_{on}$, where e is the total oxidase concentration [4–6]. Thus the pH dependent step(s) of the oxidase mechanism giving rise to the slope effect (Fig. 2) may be further specified as being due to an effect on k_{on}.

Several functional intermediates in the reaction of cytochrome oxidase with oxygen have been trapped and characterized at low temperatures [18]. Apparently the first intermediate formed is an oxy compound, compound A, which is spectrally similar to the CO-complex of the oxidase. The next event which has been identified is an oxygen reduction reaction whereby compound B, possibly a peroxy compound, is formed [18]. In a discussion of the effect of pH on the reaction between oxygen and the oxidase it is of interest to try to distinguish between the binding reaction and the oxygen reduction reaction.

By analogy with the Bohr effect on oxygen binding of hemoglobin it may be assumed that any protonization of cytochrome oxidase which has an effect on oxygen binding will have a similar effect on CO binding [19]. Thus an investigation of the pH dependence of CO binding to the oxidase could provide an indication about whether or not the binding of oxygen depends on pH. Such studies have previously been performed with rat liver mitochondria [11]. The CO-dissociation constant was not pH dependent in the presence of uncoupler and this result is confirmed by experiments with purified cytochrome oxidase. When dithionite reduced oxidase is titrated with small aliquots of CO containing buffer as described in Ref. 11 (results not shown) a plot with a Hill coefficient close to 1.0 is obtained. Within the accuracy of the method (± 0.1 μM of CO). The dissociation constant (K_D = 0.4 μM) is independent of pH between pH 5.9 and pH 8.4.

The conclusion drawn from these CO binding experiments that the actual oxygen binding is probably not affected by protonization is further supported by the observation [20] that the transient formation of compound A or the CO complex seems not to depend on pH. The following simple model for the oxidative half reaction can account for these observations:

$$E + O_2 \overset{K}{\underset{\leftarrow}{\rightarrow}} E_A \overset{k_2}{\rightarrow} E_B$$

In this model which assumes a rapid equilibration between O_2 and the reduced enzyme, E, giving rise to Compound A, E_A, the oxidation reaction is still proportional to $[O_2]$ provided $K >> [O_2]$. An apparent second order constant for the oxidation $k_{on} = k_2/K$ is obtained under these conditions [16]. It is suggested that pH has an effect on k_2 but not on K. Electron transport within the cytochrome *c* oxidase complex is in fact pH dependent since it has been found that pH has an effect on the transient formation of compound B [20] and that the midpoint potentials of cytochrome *a* and a_3 are both pH dependent [21–23].

Whether the reaction with oxygen is influenced by energization is a

matter of controversy. The effect of pH on the oxygen kinetics of the de-energized system (Fig. 2) could indicate that at least an indirect effect is possible. Energization of mitochondria and cytochrome *c* oxidase containing liposomes will result in an alkalization of the interior. Provided the reaction with oxygen takes place in an environment influenced by the internal pH it is expected that energization will have the same effect on the slope of the oxygen kinetics as an alkalization of the de-energized system. An increase in slope equivalent to a decreased on-constant is expected. The fact that the oxygen kinetics of cytochrome oxidase in its energized state does not follow a simple hyperbolic saturation curve does not allow for a direct estimate of k_{on} from the Lineweaver-Burk plot (Fig. 1). But generally the slope at a given oxygen concentration is higher with the same system in its energized state than in its de-energized state, suggesting a decrease in the apparent on-constant for oxygen upon energization.

The effect of energization on the transient oxygen kinetics of coupled mitochondria at low temperatures has been studied by Harmon and Wikström [24]. They observed that addition of ATP prior to cooling caused an acceleration in the transient formation of compound A. The addition of ATP also results in an acceleration of the reaction with CO at low temperatures [25] and increases the affinity of coupled mitochondria for CO at room temperature [11]. These observations have been interpreted to mean that the on-constant for oxygen is increased on energization [24], as opposed to the apparent decrease on energization found here and in previous communications [7,12]. The reason for this apparent controversy is not clear. It is possible that reversed electron transfer from cytochrome a_3 to cytochrome *c* upon energization could cause an enhancement of Compound A transient formation just as this effect can explain the non hyperbolic steady-state oxygen kinetics of energized proteoliposomes (Fig. 1 and Ref. 7) and mitochondria [12,26,27].

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