Cytochrome *c* Oxidase as an Electron-Transport-Driven Proton Pump: pH Dependence of the Reduction Levels of the Redox Centers during Turnover†

Per-Eric Thörnström, Peter Brzezinski, Per-Olof Fredriksson,‡ and Bo G. Malmström*

Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Göteborg, S-412 96 Göteborg, Sweden

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ABSTRACT: The pH dependence of the steady-state kinetic parameters of cytochrome oxidase has been determined in the pH range 5.4–8.4 with the enzyme in detergent solution at high ionic strength. The catalytic constant increases continuously with decreasing pH, whereas the specificity constant for reduced cytochrome *c* is essentially unchanged. The effect of pH on the aerobic transient kinetics has also been investigated in two types of experiments in a stopped-flow apparatus. In one series, a 20-fold molar excess of reduced cytochrome *c* was the only reducing substrate, whereas in the other an excess of ascorbate was used together with a mediator and varying concentrations of cytochrome *c*. In both sets the time course of the reduction levels of cytochrome *c*, cytochrome *a*, and Cu_A was monitored at specific wavelengths. In the first type of experiment, the reoxidation of cytochrome *a* was slower than cytochrome *c* oxidation. In the second type, four kinetic phases were observed, including a long steady state. The time courses, including these features, were simulated on the basis of a mechanistic model for cytochrome oxidase as a proton pump. In this model the enzyme exists in two conformations, E_1 and E_2. The intramolecular electron transfer from the primary electron acceptors to the dioxygen-reducing site is rapid in E_2 only. The transition from E_1 to E_2 is triggered by the reduction of cytochrome *a* and Cu_A. For the conversion to be rapid, the enzyme must also be doubly protonated. One of the protonation steps is rate-limiting for the entire cycle, and the pH dependence of the kinetics can be completely ascribed to the effect of proton concentration on the rate of this step.

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) catalyzes a vectorial reaction in which the energy provided by the catalytic electron transport from ferrocytochrome *c* to dioxygen is used to translocate protons across the inner mitochondrial membrane (Wikström et al., 1981). The mechanism of such electron-transport-driven pumps entails a number of basic requirements, as summarized in the cubic model of Wikström et al. (1981) and also emphasized by Malmström (1985) and by Blair et al. (1986a). One obligatory property is redox-linked conformational changes providing an alternating access of the proton-translocating group to the two sides of the membrane. To couple the catalytic driving reaction to the proton translocation, it is also necessary that the input and output of electrons take place in different conformations. Furthermore, the conformational transitions should be allowed in one direction in the protonated state only and in the reverse direction in the unprotonated state only. The binding and release of protons in the pump cycle may involve a thermodynamic linkage between protolysis and the redox change, but this is not an obligatory feature.

Any hypothetical pump mechanism should not only incorporate the basic requirements just described, but it must also satisfy the known kinetic properties of the oxidase, for example, the fact that the catalytic constant (k_0)[1] is strongly dependent on pH, whereas the specificity constant (k_s) is not. Brzezinski and Malmström (1987) have recently proposed a detailed reaction cycle for cytochrome oxidase as a proton pump, which satisfies these criteria. This cycle represents an extension of an earlier scheme (Malmström & Andréasson, 1985; Brzezinski et al., 1986) that considered only the catalytic reaction but not the proton pump. Salient features of the proposed pump mechanism are the following: (1) The enzyme can exist in two conformations, E_1 and E_2, representing the input and output states for electrons and protons. (2) Rapid electron transfer from the primary electron acceptors (cytochrome *a* and Cu_A) to the dioxygen-reducing site (cytochrome a_3–Cu_B) is possible in the E_2 state only. (3) The transition from E_1 to E_2 is rapid only if both cytochrome *a* and Cu_A are reduced and the enzyme is doubly protonated.

In this paper we describe kinetic experiments designed specifically to provide additional critical tests of our mechanism. The pH dependence of the reduction levels of cytochrome *c*, cytochrome *a*, and Cu_A during turnover has been studied in two types of stopped-flow experiments, one with cytochrome *c* as the only reducing substrate, the other with excess ascorbate and TMPD.[2] In addition, the pH dependence of k_0 and k_s has been determined in steady-state experiments. In simulating the kinetics on the basis of our scheme, we have used a single set of rate constants at all pH values in all three sets of experiments, so that all changes in rates and reduction levels are due to the effect of proton concentration on the rates of protonation and deprotonation steps. This severe restriction has the result that the fit between the simulated and experimental curves is not entirely satisfactory. All transient features

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‡ Present address: The College in Karlstad, Box 9501, S-650 09 Karlstad, Sweden.

[1] In presenting results from steady-state kinetic experiments, we use the catalytic constant k_0 (often designated k_{cat}) and the specificity constant k_s (i.e., k_0 divided by the Michaelis constant) as the constants in the Michaelis–Menten equation, following the recommendations of the Nomenclature Committee of the International Union of Biochemistry [see *Biochem. J.* (1983) *213*, 561–571].

[2] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TMPD, tetramethyl-*p*-phenylenediamine.

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are, however, well reproduced, and all changes with pH are semiquantitatively predicted. This is a strong indication that the essential features of our mechanism are correct.

Materials and Methods

Cytochrome oxidase from beef heart was isolated essentially according to the method of Van Buuren (1972). Cytochrome *c* was prepared by the procedure described by Brautigan et al. (1978) and then further purified by ion-exchange chromatography. Stock solutions of reduced cytochrome *c* were prepared by the addition of dithionite; excess dithionite was removed by gel filtration on a column of Sephadex G-25.

The difference absorbance coefficients (reduced-oxidized) used for the determination of concentration and the degrees of reduction of the redox centers were 21.1 mM^{-1} cm^{-1} at 550 nm and −3.5 mM^{-1} cm^{-1} at 563 nm for cytochrome *c*, 24 mM^{-1} cm^{-1} at 605 nm for cytochrome oxidase, 80% of the absorbance change being attributed to cytochrome *a*, and −2.5 mM^{-1} cm^{-1} at 830 nm, at which wavelength it was assumed that only this center changes absorbance on reduction, for Cu_A (Beinert et al., 1980). In the determinations of the absorbance changes of cytochrome *a* and Cu_A, corrections were made for the contributions from cytochrome *c* at 605 and 830 nm, respectively; the contributions from cytochrome oxidase at 550 and 563 nm were negligible, as cytochrome *c* was always in excess.

The buffers used in all experiments were 50 mM Hepes or Mes, depending on the pH, with 0.5% Tween 80 and 0.167 M K_2SO_4, which gives an ionic strength of 0.5 M.

Three types of kinetic experiments were carried out, two of them at 23 °C in a stopped-flow apparatus previously described (Andréasson et al., 1972; Brzezinski et al., 1986).

Steady-State Kinetics. The steady-state kinetic parameters k_0 and k_s were determined by the spectrophotometric method, as described in detail by Thörnström et al. (1984). The concentration of cytochrome oxidase was ca. 20 nM, and six concentrations of cytochrome *c* in the range 5–300 μM were employed. The initial velocities were estimated by logarithmic regression, and the kinetic parameters were determined from Hanes plots with the aid of linear regression.

Limited-Turnover Experiments. Reduced cytochrome *c* was mixed with cytochrome oxidase in the stopped-flow apparatus, and the reaction was followed at 550, 605, and 830 nm for 5 s. Separate experiments on a time scale of 200 ms at the two shorter wavelengths were performed to determine the second-order rate constant k_1 for the reaction between reduced cytochrome *c* and oxidized cytochrome *a*. In the experiments with resting oxidase, a small amount of ascorbate was present in the solution of cytochrome *c* to keep this fully reduced. To prepare the pulsed enzyme, cytochrome *c*, cytochrome oxidase, and ascorbate were mixed in one syringe and left to react until all oxygen was consumed. The reaction was then started by mixing with air-equilibrated buffer from the other syringe.

Experiments with Ascorbate and TMPD. In experiments with pulsed oxidase, the enzyme was mixed with the system ascorbate–TMPD–cytochrome *c* in one syringe, and the mixture was allowed to react until all oxygen had been exhausted. The reaction was then started by mixing with air-equilibrated buffer from the other syringe in the stopped-flow apparatus. In experiments with resting oxidase, the enzyme was kept in one syringe and the three remaining components were kept in the other. Both sets of experiments were carried out at pH 7.0 and 8.0 with observations at 550 or 563, 605, and 830 nm. The rate of cytochrome *c* reduction by ascorbate–TMPD was measured in separate experiments.

Simulations. The steady-state rate equation, in terms of all the rate constants of our complete reaction scheme (Brzezinski & Malmström, 1987), was derived on a personal computer (Ericsson PC) by the method of King and Altman (1956). Simulations of the progress curves derived from the stopped-flow experiments were carried out on an IBM 3081 computer, which solved the kinetic equations by stepwise integration with an extended Runge–Kutta method.

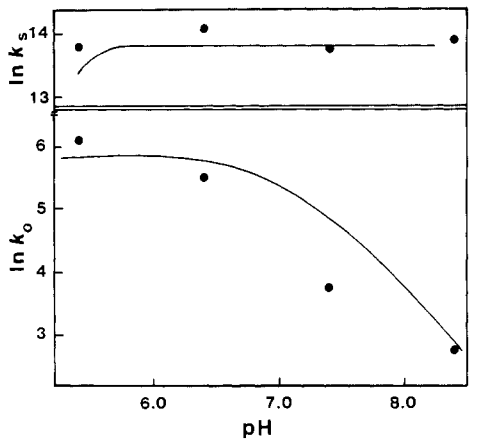

FIGURE 1: Effect of pH on the steady-state kinetic parameters k_0 and k_s in eq 1. The points are experimental, whereas the curves are simulated on the basis of the reaction cycle in Figure 4 with the rate constants in Table II.

Results

Effect of pH on the Steady-State Kinetic Parameters. The steady-state rate equation for cytochrome oxidase at high ionic strength has the simple Michaelis–Menten form (Sinjorgo et al., 1986)

$$\frac{v_0}{[E]} = \frac{k_0 k_s [S]}{k_0 + k_s [S]} \quad (1)$$

where v_0 is the initial velocity, [E] the total concentration of cytochrome oxidase, and [S] the concentration of reduced cytochrome *c*. The effect of pH on k_0 and k_s is shown in Figure 1, which also includes simulated curves, determined from the rate equation derived for our reaction cycle as described in a later section. The increase in k_0 with decreasing pH and the near constancy of k_s are in agreement with more extensive results obtained with the oxidase in detergent solution at different ionic strengths (Wilms et al., 1980) or with the enzyme reconstituted into phospholipid vesicles (Thörnström et al., 1984).

Effect of pH on the Aerobic Cytochrome c–Cytochrome Oxidase Reaction. Stopped-flow traces for the reaction between pulsed cytochrome oxidase and reduced cytochrome *c* at four different pH values are shown in Figure 2; the concentrations used allowed five complete turnovers of the enzyme. The traces for Cu_A (not shown) followed similar time courses, but the degree of reduction was always lower than that of cytochrome *a*. When the same experiments were performed with the resting form of the enzyme, two significant differences were observed: the oxidation of cytochrome *c* was slower, and the maximum reduction levels of cytochrome *a* were higher.

From the burst phase measured on a shorter time scale, it was estimated that k_1, the second-order rate constant for the reaction between cytochrome *c* and cytochrome *a*, is (2.0 ± 0.2) × 10^6 M^{-1} s^{-1} at all pH values except 5.4, where it was found to be 1.3 × 10^6 M^{-1} s^{-1}. This has as a result that the oxidation of cytochrome *c* is fastest at pH 6.4, despite the fact that k_0 is slightly higher at pH 5.4 (Figure 2).

Oxygen intermediates are expected to be present in this type of experiment (Andréasson et al., 1972). An attempt to es-

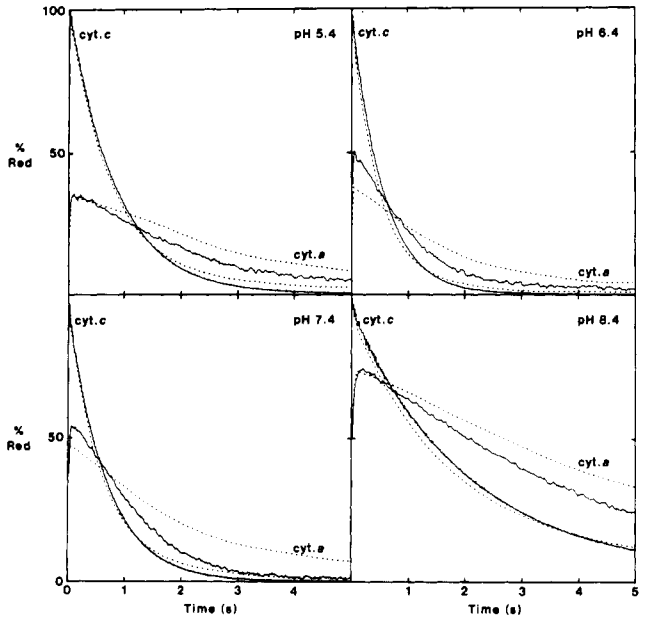

FIGURE 2: Effect of pH on the time course of the degree of reduction of cytochrome *c* and cytochrome *a* in limited turnover experiments with pulsed cytochrome oxidase. The concentrations after mixing were 2 μM oxidase, 40 μM reduced cytochrome *c*, and 137 μM O_2. The solid traces are experimental, whereas the dashed ones are simulated.

timate the uncertainty due to spectral contributions from such intermediates was made by allowing the mixed-valence carboxycytochrome oxidase to react with dioxygen in a recording spectrophotometer, which should convert the enzyme into the peroxide compound. This was found to give a positive spectral contribution at 605 nm corresponding to 6% of the absorbance change on full reduction of cytochrome *a*. At 830 nm the positive contribution was 18% of the absolute value of the change caused by reduction of Cu_A, which introduces a rather large systematic error in the estimation of the degree of reduction of Cu_A; this is one reason why the 830-nm data are not included in Figure 2.

Effect of pH on the Kinetics of Ascorbate–TMPD. When pulsed cytochrome oxidase is allowed to react with ascorbate in excess over dioxygen in the presence of cytochrome *c*–TMPD, four kinetic phases are observed, as shown by experimental and simulated traces in Figure 3, in agreement with earlier results (Brunori et al., 1979). First, there is a rapid oxidation of cytochrome *c* with a concurrent reduction of cytochrome *a* and Cu_A. This is followed by a certain amount of reoxidation of cytochrome *a* and Cu_A to steady-state reduction levels. The steady-state phase lasts until all dioxygen has been consumed, when cytochrome *c* and all redox centers of the oxidase become rapidly reduced. When the same experiment was performed with resting oxidase, there was the same initial overshoots in the reduction levels as with the pulsed enzyme, followed by a slower shift in these levels due to the conversion from the resting to the pulsed form. This demonstrates that the overshoots are not related to the resting-pulsed transition.

The type of experiment illustrated in Figure 3 was carried out with three different concentrations of cytochrome *c* at pH 7.0 and 8.0; pH values below 7 were not used because of the very slow reduction of cytochrome *c* by ascorbate–TMPD. The experimental and simulated reduction levels of cytochrome *c*, cytochrome *a*, and Cu_A are presented in Table I. This table also includes the observed steady-state times, which are compared with the times estimated from the simulations and those calculated from the steady-state kinetic equation (see next section).

Reaction Cycle and Simulations. The simulations given together with the experimental results from the three distinct types of kinetic experiments in Figures 1–3 and Table I have all been made on the basis of the previously described reaction cycle for cytochrome oxidase as a proton pump (Brzezinski & Malmström, 1987). As reaction steps that are very rapid compared to all other steps do not influence the simulations, the simplified scheme given in Figure 4 has actually been used. Two simplifications have been introduced and will be justified: (1) The binding of one proton only in the E_1 state (step 7) and its dissociation in the E_2 state (step 9) have been included; (2) All steps following the conformational transition (step 8) and proton dissociation (step 9), leading to the formation of the fully oxidized enzyme in the E_2 state, have been combined into one step (step 10).

To simulate the pH dependence observed in all three types of experiments, one of the two proton-binding groups must have a low pK_a value in the E_1 state, in accord with the transition-state mechanism (Malmström, 1985), whereas the

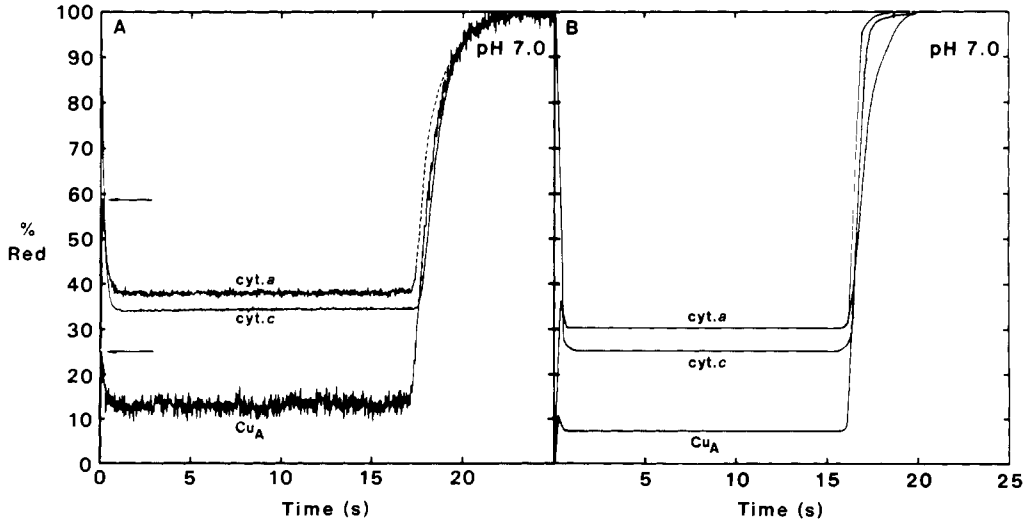

FIGURE 3: Effect of pH on the time course of the degree of reduction of cytochrome *c*, cytochrome *a*, and Cu_A in experiments with pulsed cytochrome oxidase reduced with ascorbate–TMPD. The concentrations in the reaction cuvette were 5 μM oxidase, 25 μM cytochrome *c*, 50 μM TMPD, 7 mM ascorbate, and 137 μM O_2. (A) Experimental traces: The arrows indicate the maximum in the overshoots for cytochrome *a* and Cu_A. The final reductive phase for cytochrome *a* is indicated by a dashed line, as cytochrome a_3 is no longer fully oxidized and contributes to the absorbance increase at 605 nm. (B) Simulated traces.

Table I: Effect of pH and Cytochrome *c* Concentration on the Reduction Levels and Steady-State Times with Pulsed Cytochrome Oxidase in Experiments of the Type Illustrated by Figure 3[a]

		reduction level[b] (%)								
		cytochrome *c*		cytochrome *a*		Cu_A		steady-state time[b] (s)		
pH	cytochrome *c* concn (μM)	exptl	sim	exptl	sim	exptl	sim	exptl	sim	calcd[c]
7.0	25	34	27	38	32	13	9	18	16	15
	50	35	30	42	34	15	11	8.7	8.0	9.4
	100	40	34	56	40	25	14	4.8	4.3	5.7
8.0	25	57	50	59	48	28	23	16	15.3	14.5
	50	64	58	69	58	37	33	9.6	9.3	9.8
	100	72	67	78	70	48	47	6.2	6.2	7.4

[a] Experimental conditions are given in the legend to Figure 3. [b] Both experimental and simulated values are given. [c] Values calculated from eq 3, as described in the text.

Table II: Rate Constants in the Reaction Cycle (Figure 4) Used in the Simulations[a,b]

	step (*i*)[c]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
k_i	exptl	10^3	10^3	10^3	1.8×10^7	10^3	10^{11}	10^3	4×10^4	exptl	2	1.8×10^7	10^3
k_{-i}	2×10^3	2.2×10^6	10^2	4×10^3	10^3	3.5×10^6	4×10^4	10^2	10^{11}		4	10^3	1.8×10^7

[a] exptl means that an experimental value has been used, as described in the text. [b] The unit of the constants is s^{-1} for unimolecular steps and M^{-1} s^{-1} for bimolecular steps. [c] The forward direction of the reactions is defined in the clockwise direction through the cycle, i.e., the same direction in which *i* increases.

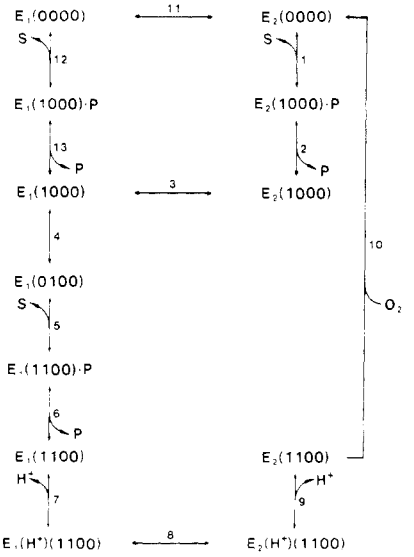

FIGURE 4: Reaction cycle for cytochrome oxidase used in the simulations. E_1 and E_2 represent two different conformations of the oxidase. The digits in parentheses designate the four redox centers in the order cytochrome *a*, Cu_A, Cu_B, and cytochrome a_3, 0 representing an oxidized center and 1 a reduced one. For a complete turnover the enzyme must go through the entire cycle twice, with peroxide bound to the cytochrome a_3–Cu_B site in the second cycle.

other group must display a thermodyanmic linkage ("membrane Bohr effect"); i.e., it should have a high pK_a in the reduced E_1 state and as low one in the oxidized E_2 state. This has as a result that the steps involving proton binding to and dissociation from the latter group have very favorable equilibria, and hence rates, in the forward reaction compared to those of the second group, for which the binding step is slowed by a factor of 100 due to the unfavorable equilibrium [see Brzezinski and Malmström (1986)]. Thus, it is only the binding of one of the protons that contributes to the rate limitation, and the other one need not be included in thesimulations (cf. Table II and the comments to it).

The two first of the combined reactions in step 10 are intramolecular electron transfers to give $E_2(0011)$, and the slowest of these two transfers has a first-order rate constant of about 10^3 s^{-1} (Brzezinski & Malmström, 1987). The next reaction in step 10 is a combination of $E_2(0011)$ with O_2, which has a second-order rate constant of 10^8 M^{-1} s^{-1} (Greenwood & Gibson, 1969), so that this step has an apparent first-order rate constant $>10^3$ s^{-1} until most of the dioxygen has been consumed. In addition, the oxygen adduct is converted to the peroxo intermediate with a half-time of about 30 μs (Brunori & Gibson, 1983), corresponding to a first-order rate constant of ca. 2×10^4 s^{-1}. Thus, it is justified to combine all these reactions into a single step 10 with a rate constant equal to that for the slowest partial reaction. This also follows from the fact that the fully reduced enzyme is completely reoxidized by dioxygen in about 1 ms with a slowest step of about 10^3 s^{-1} (Greenwood & Gibson, 1969), as this corresponds to step 10 in the second cycle with peroxide bound to cytochrome a_3–Cu_B. The great rapidity of the dioxygen reaction is, in addition, demonstrated by the fact that the steady-state levels in Figure 3 are independent of the concentration of dioxygen until the point of exhaustion of this substrate.

The rate constants used in all the simulations of the results in Figures 1–3 and Table I are given in Table II, and they may require some comments. Experimental values are used for k_1 and k_{10} (see above). In the simulations in Figure 3 and Table I, experimental values have also been employed for the rate of reduction of cytochrome *c* by ascorbate–TMPD. No first-order rate constant in the forward direction has been allowed to be smaller than 10^3 s^{-1}, as otherwise k_0 could not reach the high values observed at low pH (Figure 1). The value for k_5, which is identical with that of k_{12}, is higher than the value of k_1, as cytochrome *a* has a higher reduction potential in the E_1 compared to the E_2 state (Brzezinski & Malmström, 1987) and as a higher driving force for the electron transfer should increase the rate (Marcus & Sutin, 1985). The pK_a of the proton-translocating group has been assumed to be 6.4, i.e., 1 unit below the pH on the cytosol side of the inner mitochondrial membrane, as required by the transition-state mechanism (Malmström, 1985). The on-rate constants in the proton equilibria (k_7 and k_9) have been assumed to be 10^{11} M^{-1} s^{-1}, and this, together with the pK_a value, gives the values of k_{-7} and k_9. The value of k_3 is larger than that of k_{11} to be consistent with the fact that under our conditions the steady-state rate equation is hyperbolic (cf. eq 1 and eq 2 below). Furthermore, the rate constants used correspond to equilibrium constants that, when multiplied together, give the equilibrium constant for the cytochrome *c*–

dioxygen reaction (approximately 10^{19}), as required by thermodynamics.

Note that the same rate constants have been used for the simulations of all the results from the three distinct types of kinetic experiments reported and that no rate constant has been assumed to vary with pH. Thus, the changes due to pH in the simulations are all attributed to changes in the proton on-rates in steps 7 and 9, which get apparent first-order rate constants of $10^{(11-pH)}$ s^{-1}. In view of the large number of rate constants, the fits can, of course, be considerably improved if some of these are varied with pH. We deemed it, however, more important to demonstrate that the major effect of pH on the kinetics can be ascribed to changes in the rate of the conformational switch in step 8, as required in the transition-state mechanism (Malmström, 1985).

The steady-state rate equation derived for the scheme in Figure 4 has the form

$$\frac{v_0}{[E]} = \frac{a[S] + b[S]^2}{1 + c[S] + d[S]^2} \quad (2)$$

in which the coefficients a–d are complicated functions of all the rate constants [cf. Brzezinski and Malmström (1986)].[3] With the rate constants given in Table II, the reaction path involving steps 11–13 is, however, much slower than that via steps 1–3. This has as a result that eq 2 reduces to the form of eq 1, as observed experimentally at the high ionic strength used in this study [see Brzezinski and Malmström (1986)]. For this reason, single simulated values of k_0 and k_s have been given in Figure 1.

The steady-state rate equation for cytochrome oxidase in the presence of both reduced and oxidized cytochrome c, as in the experiments of Figure 3 and Table I, is (Minnaert, 1961)

$$\frac{v}{[E]} = \frac{k_0 k_s[S]}{k_0 + k_s([S] + [P])} \quad (3)$$

The steady-state levels for cytochrome c in Table I give the concentrations of S and P, and these values, together with the experimental values of k_0 and k_s (Figure 1), have been used for the calculation of the steady-state times.

Discussion

The mechanistic scheme for cytochrome oxidae (Brzezinski & Malmström, 1987), which we have tried to test by the kinetic experiments described in this paper, is an extension of a reaction cycle proposed for the catalytic electron transfer at constant pH (Malmström & Andréasson, 1985; Brzezinski et al., 1986). The main changes compared to the earlier scheme are the introduction of two conformations, E_1 and E_2, and protonation–deprotonation steps. Both additions are necessary if cytochrome oxidase shall function as a proton pump, and they are also supported by known kinetic properties of the enzyme. Thus, the involvement of two conformations can account for the nonhyperbolic steady-state kinetics observed at low ionic strength (Brzezinski & Malmström, 1986), and the protonic equilibrium leads to the pH dependence of k_0 and k_s found experimentally, as demonstrated by the simulations in Figure 1. It should be noted that the oxidase must go through the conformational transitions and protonation–deprotonation steps also in the uncoupled state, as in a pump there must be an obligatory coupling between the electron-transfer and the ion-translocation reactions (Malmström, 1985). This has the consequence that many features of the pump mechanism manifest themselves also in the kinetic behavior of the solubilized enzyme, and this provides the rationale behind the present study.

The similarity between the experimental observations (Figures 1–3 and Table I) and the simulations based on the reaction sequence in Figure 4 provides support for our mechanistic scheme. Even if the fit is only semiquantitative, all qualitative features are well reproduced. For example, k_0 increases with decreasing pH (Figure 1), and the rate of oxidation of cytochrome c at constant concentration has, despite this, a maximum at pH 6.4 (Figure 2). The reduction levels of cytochrome a in Figure 2 change in the same way with pH in the experimental and simulated traces. In the experiments of the type illustrated in Figure 3, the order of the cytochrome c, cytochrome a, and Cu_A levels are well reproduced, and they all change with cytochrome c concentration and pH in the manner predicted (Table I). That there are discrepancies between experiments and simulations is not surprising, as our scheme undoubtedly is oversimplified. Except for the first electron added, we have a linear electron-transfer sequence, but branches are expected at the points of the E–P complexes [cf. Brzezinski et al. (1986)]. Furthermore, we have assumed that all steps have the same rate constants in both cycles of the complete reaction, but the rates of some steps are probably enhanced by the greater driving force in the peroxo intermediate of the second cycle. Finally, we have not introduced any corrections for the spectral contributions of oxygen intermediates, and we have made the rather unrealistic assumption that all rate constants (except k_1) are entirely independent of pH.

In discussing some individual steps of our mechanism in greater detail, we will refer to the simplified scheme in Figure 4; the differences between this and the complete scheme have already been outlined. The value of k_s is 1.1×10^6 M^{-1} s^{-1}, which is very close to the experimentally observed value of k_1. This has the consequence that at low substrate concentration (where $k_s[S] \ll k_0$ in eq 1) the reaction is essentially diffusion-controlled. Thus, cytochrome oxidase is a "perfect enzyme", as defined by Albery and Knowles (1976).

It is commonly assumed that the Michaelis constant (K_m) for cytochrome oxidase reflects its affinity for cytochrome c; the terms high- and low-affinity K_m are, for example, used to describe the nonhyperbolic steady-state kinetics [see Sinjorgo et al. (1987)]. Our results show that this is incorrect, as the affinity (k_1/k_{-1}) is almost independent of pH, whereas K_m increases linearly with k_0 and thus is very large at low pH. This follows from the fact that k_s is constant when k_0 increases (Figure 1), because $K_m = k_0/k_s$.

We have assumed that the primary acceptor of electrons from cytochrome c is cytochrome a. Our results are, however, equally consistent with Cu_A being the primary acceptor, as there is a rapid redox equilibrium between cytochrome a and Cu_A. This is demonstrated by the observation that these two redox centers are reduced with the same speed in the initial burst phase of the reaction between cytochrome c and cytochrome oxidase [see also Antalis and Palmer (1982)]. The equilibrium constant we have to use for step 4 in the simulations is 0.25, and this favors cytochrome a as the primary acceptor, because this has a slightly higher reduction potential than Cu_A, particularly in the pulsed state (Brunori et al., 1979).

The value used for k_2 and k_6 (10^3 s^{-1}) in consistent with the fact that product dissociation must be fast, not rate-limiting as often assumed. This follows from the observation of Smith and Conrad (1956) that cytochrome c oxidation is first order,

[3] Because of its very cumbersome form, the complete equation is not presented here. A computer printout of the equation and its derivation can be requested from the authors.

not zero order, even at the highest substrate concentrations. It is also evidenced by the partial reduction of the primary electron acceptor (cytochrome *a* or Cu_A) seen in the turnover experiments in Figures 2 and 3 [cf. Brzezinski et al. (1986)].

An important part of our mechanism is the requirement that both cytochrome *a* and Cu_A must be reduced before internal electron transfer to the dioxygen-reducing site can occur. This was first concluded from the finding that, in limited turnover experiments, cytochrome *a* is still partially reduced when all cytochrome *c* has been oxidized (Antonini et al., 1970; Brzezinski et al., 1986; Fabian et al., 1987), as also seen in Figure 2 (pH 5.4). Copeland et al. (1987) have suggested that an apparent two-electron requirement for the conformational change and heme electron transfer is obtained because reduction of Cu_A induces the change, whereas the redox equilibrium is toward cytochrome *a*. We still favor a true two-electron requirement, however, because all other enzymes reducing dioxygen to water show this requirement (Malmström, 1982), probably in order to avoid the formation of significant amounts of oxygen radical intermediates.

To maintain the observed H^+/e^- stoichiometry in the proton translocation (Wikström et al., 1981), the enzyme must also be doubly protonated for the transition from the E_1 to the E_2 state (step 8) to be rapid. We have assumed that it is these protons which are responsible for the strong pH dependence of k_0. As an alternative it has been suggested (Wilms et al., 1980) that the dependence is related to the protons involved in dioxygen reduction, but this is excluded by the fact that the slowest step in this reaction has a rate constant 10^3 s^{-1}, which is much higher than k_0 even at high pH (Greenwood & Gibson, 1967). As already discussed, we must assume that one proton is bound to a group with a constant, low pK_a of 6.4, as required in the transition-state mechanism (Malmström, 1985), whereas the other proton-binding group displays an appreciable Bohr effect and has been left out in the simulations. An alternative, which is perhaps more reailistic, would be the association of a weak Bohr effect with both protons. Combined with the two-electron requirement, this would nicely account for the moderate pH dependence (−30 mV/pH) in the reduction potential of cytochrome *a* (Blair et al., 1986b). A good simulation of the pH dependence of k_0 must, however, involve three protons (Wilms et al., 1980; Thörnström et al., 1984).

It has earlier been concluded that the rate-limiting step in the cytochrome oxidase reaction is the internal electron transfer to the dioxygen-reducing site (Brunori et al., 1982; Brzezinski et al., 1986; Bickar et al., 1986). Recently it was, however, shown that this electron transfer is rapid in the E_2 state, and it was suggested that it is the $E_1 \rightarrow E_2$ transition which is rate-limiting (Brzezinski & Malmström, 1987). The rate constants in Table II show that it is not the conformational transition (step 8) per se which is rate-limiting, as this is rapid in the protonated state, but rather the protonation in step 7. This is slow because of the low pK_a, which has the consequence that the back reaction is more rapid than the forward reaction at high pH. It is then also the effect of the proton concentration on the rate of step 7 that is responsible for the pH dependence of k_0.

It is easy to visualize a molecular basis for the demand that the enzyme should be doubly protonated for step 8 to be rapid. The tertiary and quaternary structures of proteins are to a large extent stabilized by electrostatic interactions (salt bridges, hydrogen bonds). The binding of protons to amino acid side chains in a protein can lead to the formation as well as the breaking of such bonds. Thus, we would like to suggest that such bond rearrangements on the protonation of two acid–base groups in cytochrome oxidase stabilizes the E_2 state relative to E_1, thereby making the free energy change in the transition of step 8 more negative. This increase in driving force on protonation would also increase the rate of the conformational change, as required by our pump mechanism.

To explain why the internal electron transfer is rapid in E_2 but not in E_1, Brzezinski and Malmström (1987) suggested that the conformational change induced by the reduction of cytochrome *a* and Cu_A, and the subsequent double protonation, is transmitted to cytochrome a_3–Cu_B in an allosteric fashion, thereby removing the reorganizational barrier for electron transfer. Also for this part of our mechanism, one can surmise a molecular basis. Holm et al. (1987) have suggested a molecular model for cytochrome oxidase, in which both heme groups are sandwiched between two transmembrane helices of subunit I. Slight movements of these helices on reduction of the primary electron acceptors could change the distance between the hemes and their relative orientation. This could in turn affect the rate of electron transfer, thus providing a molecular mechanism for electron gating in cytochrome oxidase.

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Absence of Hepatic Cytochrome P450bufI Causes Genetically Deficient Debrisoquine Oxidation in Man†

Ulrich M. Zanger,‡ Francis Vilbois,‡ James P. Hardwick,§ and Urs A. Meyer*,‡
Department of Pharmacology, Biocenter of the University of Basel, CH-4056 Basel, Switzerland, and Biomedical Division, Argonne National Laboratories, 9700 S. Cass Avenue, Argonne, Illinois 60439
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ABSTRACT: The common genetic deficiency of drug oxidation known as debrisoquine/sparteine-type polymorphism was investigated with bufuralol as prototype substrate. In human liver microsomes the 1′-hydroxylation of bufuralol is catalyzed by two functionally distinct P-450 isozymes, the high-affinity/highly stereoselective P450bufI and the low-affinity/nonstereoselective P450bufII. We demonstrate that P450bufI is unique in hydroxylating bufuralol in a cumene hydroperoxide (CuOOH) mediated reaction whereas P450bufII is active only in the classical NADPH- and O_2-supported monooxygenation. In microsomes of liver biopsies of in vivo phenotyped poor metabolizers of debrisoquine or sparteine, the CuOOH-mediated activity was drastically reduced. Rabbit antibodies against a rat P-450 isozyme with high bufuralol 1′-hydroxylase activity (P450db1) precipitated exclusively P450bufI-type activity from solubilized microsomes. Western blotting of microsomes with these antibodies revealed a close correlation between the immunoreactive protein and CuOOH-mediated (+)-bufuralol 1′-hydroxylation. No immunoreactive protein was detected in liver microsomes of in vivo phenotyped poor metabolizers. These data provide evidence for a specific deficiency of P450bufI and are consistent with the complete or almost complete absence of this protein in the liver of poor metabolizers.

Cytochrome P-450 (P-450)[1] is the collective term for a group of hemoprotein isozymes with broad and overlapping substrate specificities responsible for the oxidative metabolism of a large number of endobiotic and xenobiotic substances (Ortiz de Montellano, 1986; Meyer, 1984; Adesnik & Atchison, 1986). In recent years, several genetically determined polymorphisms of P-450-mediated drug oxidation have been discovered (Idle & Smith, 1979; Küpfer & Preisig, 1983; Eichelbaum, 1984). These polymorphisms cause impaired biotransformation of certain drugs in so-called "poor metabolizer" or PM subjects. Because of their frequency of occurrence, genetic polymorphisms of drug oxidation are a major determinant of interindividual differences in the therapeutic and toxic responses to numerous clinically important drugs.

One of the most extensively studied examples of a genetically determined variation in drug metabolism is the debrisoquine/sparteine-type polymorphism which occurs in up to 10% of individuals in Caucasian populations and affects the metabolism of over 20 clinically used drugs including debrisoquine, sparteine, and bufuralol (Price-Evans, 1986; Meyer et al., 1986). Pedigree studies suggest that this defect is monogenically inherited as an autosomal recessive trait (Eichelbaum et al., 1979; Price-Evans et al., 1980). Numerous studies have been undertaken to evaluate the molecular basis of this important polymorphism, but so far without success.

Investigations of the metabolism of debrisoquine or bufuralol in microsomes of liver biopsies of in vivo phenotyped extensive and poor metabolizer subjects have supported the hypothesis

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* To whom correspondence should be addressed.
‡ Biocenter of the University of Basel.
§ Argonne National Laboratories.

[1] Abbreviations: P-450, cytochrome P-450; CuOOH, cumene hydroperoxide; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate; EM, extensive metabolizer (phenotype); PM, poor metabolizer (phenotype); KDL, kidney donor liver.

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