Studies on Cytochrome Oxidase

VI. KINETICS OF THE AEROBIC OXIDATION OF FERROCYTOCHROME c BY CYTOCHROME OXIDASE*

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The electron transfer reaction catalyzed by cytochrome oxidase can be represented by Equation 1.

\[
\text{Ferrocytochrome } c + \frac{1}{2}O_2 + H^+ \xrightarrow{\text{cytochrome oxidase}} \text{ferri} \text{cytochrome } c + \frac{1}{2}H_2O
\]

The rate of reaction can be determined by measuring the rate of either oxygen consumption or ferrocytochrome c oxidation. When the rate of oxygen consumption is measured either manometrically or polarographically, a mixture of a limited amount of cytochrome c and an excess of reducing agent, instead of excess ferrocytochrome c, is used as an electron donor system. In this case the true electron donor, ferrocytochrome c, is constantly regenerated through the chemical reduction of ferriacytochrome c. This system yields a usual hyperbolic relationship between rate and cytochrome c concentration as first reported by Stotz, Altschul, and Hogness (6) and confirmed by subsequent workers (2, 4, 7, 8). Further analyses of this system, however, reveal several facts which seem kinetically anomalous; rates were not always proportional to the enzyme concentrations and \( K_m \) values for cytochrome c were greatly dependent on concentrations of reducing agent and enzyme (2, 4, 7). Although the complete reduction of cytochrome c during the rate measurement is one of the prerequisite conditions for the kinetic analysis of this system, spectrophotometric examinations (4, 8) revealed that cytochrome c was not always fully reduced under the experimental conditions at which this system had been studied. This imposes an additional complication in the kinetic analysis. For example, the addition of an enzyme inhibitor to such a system may induce two opposite effects; namely, a rate-suppressing effect due to a decrease in the effective enzyme concentration and a rate-stimulating effect due to an increase in the concentrations of substrate (ferrocytochrome c) and product (ferriacytochrome c), respectively. This results in the increased steady state reduction of cytochrome c as well documented by the “crossover” theorem of Chance and Williams (9). Without eliminating the latter rate-stimulating effect, it was predicted that kinetic analyses of such a system would have resulted in anomalous observations as mentioned above (4, 7, 8).

The spectrophotometric measurement of the oxidation rate of ferrocytochrome c in the absence of reducing agents is a more direct method of assay. Since the time course of the reaction in this spectrophotometric system was found to obey first order kinetics with respect to the ferrocytochrome c concentration, the cytochrome oxidase activity measured by this method has been expressed in terms of first order rate constants (10–15). Smith and Conrad (10) studied the kinetics of this system extensively and found that first order rate constants were independent of the ratio of ferro- to ferriocytochrome c but decreased upon increasing the concentrations of total cytochrome c. They considered that these phenomena were due to the inhibitory effects of both ferro- and ferriocytochrome c, although they did not discuss the theoretical basis for such an interpretation in detail. Despite their clear demonstration of the inhibitory effect of ferrocytochrome c, it was theoretically impossible to determine the inhibitor constant of ferrocytochrome c as long as the enzyme activity was expressed in terms of a first order rate constant. It is generally difficult to derive equations which satisfactorily represent the progress curves of enzyme reaction, because of the numerous factors which may alter the velocity during the course of the reaction, as pointed out by Dixon and Webb (16). In fact, some workers (12, 14, 17–19) have tried to formulate rate equations to explain the kinetics of this enzyme reaction, but this was possible only after making a series of assumptions which have yet to be proven.

In the present paper, the aerobic oxidation of ferrocytochrome c catalyzed by cytochrome oxidase has been kinetically analyzed through rate measurements at initial steady states, the theory of which has been firmly established from the mathematical as well as experimental points of view in a number of enzyme systems (17). The measurements have been made in the presence and absence of inhibitors of cytochrome oxidase. On the basis of determined kinetic constants, rate equations for the present assay system have been formulated. These equations, which were originally worked out by Harmon and Niemann (20), seem to conform to the experimental results of both initial steady state kinetics and time course kinetics of the cytochrome oxidase reaction.

MATERIALS AND METHODS

Enzyme Preparations—A purified preparation of cytochrome oxidase, which will be hereafter referred to as the purified oxidase, was made from beef hearts according to the method described elsewhere (1, 3, 21). This preparation was dissolved in 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Tween 80 and stored at 0°C. A particular preparation of heart muscle was made according to a slight modification of the Keilin-Hartree method (22). The solubilized particulate prep-
aration was a supernatant obtained by a high speed centrifugation of the particulate preparation which had been treated with 2% cholate. The concentration of cytochrome oxidase in these preparations was determined spectrophotometrically from the absorbance difference between reduced and oxidized preparations at 565 nm on the basis of an extinction coefficient for heme a of 11.0 m\(^{-1}\) cm\(^{-1}\) (3, 21).

**Cytochrome c**—Lyophilized cytochrome c (type III from horse hearts; 95% pure) was purchased from Sigma Chemical Company and used without further purification. Ferrocytochrome c was prepared by an anaerobic gel filtration of a dithionite-reduced cytochrome c (21). The lyophilized cytochrome c, 110 mg, was dissolved in 2 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and saturated with nitrogen. The solution was reduced by adding a minimal amount of solid sodium dithionite. The reduced cytochrome c solution was placed on the top of a column (10 \times 250 mm) of Sephadex G-25 (fine grain beads obtained from Pharmacia, Uppsala, Sweden) which had been equilibrated with the same buffer saturated with nitrogen. The gel filtration was carried out under a minimal hydrostatic pressure to obtain a slow flow rate which resulted in a maximal separation of ferrocytochrome c from excess dithionite and its reaction products. The middle three-fifths (about 2 ml) of the deeply pink-red fraction were collected. The cytochrome c (about 2 mg) so prepared was usually 96 to 98% reduced. The rate of autoxidation was about 1% per day at 0° even under aerobic conditions. The concentration of cytochrome c was determined spectrophotometrically with the following extinction coefficients (22): \(\varepsilon_{260nm}(\text{reduced})\) 27.7 m\(^{-1}\) cm\(^{-1}\) and \(\Delta\varepsilon_{260nm}(\text{reduced minus oxidized})\) 18.5 m\(^{-1}\) cm\(^{-1}\). Ferrocytochrome c was prepared similarly by an aerobic gel filtration of cytochrome c treated with excess potassium ferricyanide.

**Buffers**—Potassium phosphate buffers of a constant ionic strength of 0.1 (pH 6.0, 6.3, 7.0, 7.4, and 8.0) were used throughout the present study.

**Instruments**—Spectrophotometric determinations were made with a Cary 15 recording spectrophotometer equipped with a thermostated cell compartment. The pH determinations were made with a Radiometer pH meter 23.

**Assay of Cytochrome Oxidase Activity**—The cytochrome oxidase activity was assayed spectrophotometrically by measuring the rate of aerobic oxidation of ferrocytochrome c catalyzed by cytochrome oxidase at 23°. The reaction was initiated by rapidly mixing 5 to 20 \(\mu\)l of 0.5 to 1.0 \(\mu\)M cytochrome oxidase into reaction mixtures (2.0 ml) containing varying amounts of ferrocytochrome c (0 to 150 \(\mu\)M). The reaction was followed by measuring the decrease in absorbance of ferrocytochrome c at 550 mg with time (cf. Fig. 1A). When 70 to 90% of ferrocytochrome c was oxidized, excess potassium ferricyanide was added to the mixture to oxidize the remaining ferrocytochrome c completely. Initial rates were calculated by measuring the decrease in absorbance of ferrocytochrome c at 550 mg with time (cf. Fig. 1A). A was obtained at normal recording conditions, while B was recorded at an expanded time and sensitivity scale to illustrate the initial portion of the reaction.

The initial rates determined by the differential and integral methods were in good agreement with each other with a maximal deviation of 10% (cf. Fig. 1). When the time course curve of the reaction did not obey first order kinetics the initial rates were

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**RESULTS**

**Kinetics in Absence of Added Inhibitors**—Fig. 1A illustrates a time course curve of the aerobic oxidation of ferrocytochrome c catalyzed by the purified oxidase at pH 7.0 recorded as the decrease in absorbance of ferrocytochrome c with time. In agreement with the conclusions of previous workers (10–16), the reaction was apparently first order with respect to the concentration of ferrocytochrome c, since the plot of \(\log[\text{ferrocytochrome c}]/\text{time}\) was a straight line. Such plots obtained with the purified oxidase were apparently straight lines at pH 6.0, 6.3, and 7.0, but slight and definite deviations from straight lines were observed at pH 7.4 and 8.0, respectively. Plots obtained with the particulate preparation were straight lines at all the pH values tested, while those obtained with the solubilized particulate preparation under the identical conditions revealed deviations from straight lines at pH 7.4 and 8.0 as shown in Fig. 2, which were similar to those obtained with purified oxidase. Such deviations could not be attributed to a progressive inactivation of the enzyme during the course of reaction, since both the purified oxidase and the solubilized particulate preparation were stable at these pH values. This problem will be discussed in detail subsequently.

The initial rates determined by the differential and integral methods were in good agreement with each other with a maximal deviation of 10% (cf. Fig. 1). When the time course curve of the reaction did not obey first order kinetics the initial rates were
determined by the former method only, since the latter method was no longer applicable. When initial concentrations of ferrocytochrome c were low, time course curves were recorded at an expanded time and sensitivity scale to ensure the accurate measurement of initial rates, as shown in Fig. 1B.

In agreement with the results of previous workers (3, 10, 15), initial rates of the aerobic oxidation of ferrocytochrome c were found to be strictly proportional to the enzyme concentration under the present conditions. The reciprocal of the initial rate was found to be related linearly to the reciprocal of the initial concentration of ferrocytochrome c at all of the pH values tested. Values of $\frac{V_{\text{max}}}{V}$ and $K_m$ for ferrocytochrome c were determined according to the Lineweaver-Burk procedure (25) and shown in Table I. It should be noted that $\frac{V_{\text{max}}}{V}$ values of solubilized oxidase such as the solubilized particulate preparation and the purified oxidase were much greater than those of the particulate preparation and were more sensitive to pH changes.

**Kinetics in Presence of Added Ferricytochrome c—**Initial rates decreased in the presence of added ferri- cytochrome c. Although this had been recognized by previous workers (10, 15), it is surprising to note that the type of inhibition and the value of $K_i$ for ferrocytochrome c have never been reported. Lineweaver-Burk plots of initial rates in the presence and absence of ferricytochrome c revealed that ferrocytochrome c inhibited cytochrome oxidase strictly competitively with ferrocytochrome c at all the pH values tested (cf. Fig. 3). $K_i$ values of ferricytochrome c were calculated from slopes of Lineweaver-Burk plots and are shown in Table I. It should be noted that $K_i$ values of ferricytochrome c and $K_m$ values of ferrocytochrome c obtained in the particulate preparation were similar at all pH values tested, while those obtained with solubilized enzymes were similar at acid and neutral pH values, but different at alkaline pH values. For example, at pH 8.0, $K_i$ values of ferricytochrome c were about one half of $K_m$ values of ferrocytochrome c in the solubilized enzyme systems.

**Formulation of Rate Equations—**On the basis of the above mentioned observations, it is possible to formulate rate equations for the aerobic oxidation of ferrocytochrome c catalyzed by cytochrome oxidase. Under usual assay conditions, namely, in air-saturated aqueous media at constant pH, the cytochrome oxidase reaction (Equation 1) was considered to be independent of $[O_2]$, $[H^+]$, and $[H_2O]$, since $[O_2] \gg K_m$ of $O_2$ (26). Thus, Equation 1 could be simplified to Equation 2.

$$
\text{Ferricytochrome c} \rightarrow \text{cytochrome oxidase} \rightarrow \text{ferrocytochrome c + e}^{-} \quad (2)
$$

Since the electron ($e^-$) is actually removed from the system by being assimilated into the water produced, ferrocytochrome c (substrate or S), ferricytochrome c (product or P), and cytochrome oxidase (enzyme or E) become the only reactants to be considered in the kinetics of the system. The rectangular hyperbolic relationship between rate and substrate concentration indicates the formation of an active intermediate (ES) during the cytochrome oxidase reaction without specifying the nature of the intermediate (cf. Equation 3).

$$
E_{\text{tot}} + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P \quad (3)
$$

Since ferricytochrome c is an inhibitor which is competitive with ferrocytochrome c, Equation 4 is formulated.

| pH  | $K_m$ | $K_i$ | $V_{\text{max}}$ | $K_m$ | $K_i$ | $V_{\text{max}}$
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$a$ $V_{\text{max}}$ = $k_1$ of Equation 3.

$b$ $K_m$ of ferrocytochrome c.

$c$ $K_i$ of ferrocytochrome c.

d $k_i = k_1 \cdot k_2 / (K_m + K_i$). If $k_i \gg k_1$, $k_i = k_1$ (see "Discussion").
by the present results of initial rate measurements in the presence and absence of an added product (p. + s0) in the presence and absence of other hydrolyzing enzymes by subsequent workers (27, 28). Under initial steady state conditions (s = s0 and p = 0) in the presence and absence of an added product (p0 ≠ 0 and p0 = 0, respectively), Equation 5 becomes familiar rate equations of the Michaelis-Menten type (29). Thus Equation 5 is satisfied by the present results of initial rate measurements in the presence and absence of added ferricytochrome c.

In order to express the time course of the reaction, Equation 5 is integrated to Equation 6 (20, 27, 28).

\[
V_{\text{max}} t = \left[ K_m + (p_0 + s_0) K_m / K_i \right] \ln \left( \frac{s_0}{s} \right) + \left( 1 - \frac{K_m}{K_i} \right) \left( s_0 - s \right)
\]

where s = substrate concentration at time = t. Since the sum of p0 + s0 remains a constant during each determination, the only condition at which Equation 6 represents the first order time course is K_m = K_i. As shown in Table I, when K_m = K_i, the time course curves of the reaction were apparently first order, while at K_m ≠ K_i (for example, with the solubilized particulate preparation at pH 8.0), time course curves deviated significantly from first order kinetics.

When first order kinetics is maintained and thus K_m = K_i is assumed, Equation 6 can be simplified to Equation 7.

\[
V_{\text{max}} t = \left[ K_m + (p_0 + s_0) \right] \ln \left( \frac{s_0}{s} \right)
\]

Equation 7 indicates that the time course of the reaction is apparently first order with respect to s. The apparent first order rate constant (k_f) is expressed in Equation 8.

\[
k_f = \ln \left( \frac{s_0}{s} \right) / t = V_{\text{max}} / \left[ K_m + (p_0 + s_0) \right]
\]

Equation 8 indicates that the apparent first order rate constant is a simple function of the sum of p0 + s0; k_f decreases upon increasing (p0 + s0), as observed by Smith and Conrad (10).

The inversion of Equation 8 gives Equation 9.

\[
1/k_f = K_m / V_{\text{max}} + (p_0 + s_0) / V_{\text{max}}
\]

Equation 9 predicts a linear relationship between 1/k_f and (p0 + s0) as shown in Fig. 4. At (p0 + s0) = 0, k_f approaches a maximal value of V_{max}/K_m. The apparent second order rate constant (k_2) can be obtained by dividing V_{max}/K_m with e.

\[
k_2 = V_{\text{max}} / K_m e = k_1 k_3 / (k_2 + k_3) \text{ at } K_m > s
\]

k_1 becomes equal to the true second order rate constant, k_2 of Equation 3, only if k_3 >> k_3 (see “Discussion”). The k_2 values are given in Table I. It is noteworthy that one can actually determine K_m and V_{max} (and thus k_2 = V_{max}/e) by measuring apparent first order rate constants at different concentrations of total cytochrome c, since the intercepts at the 1/k_f and (p0 + s0) axes are K_m/V_{max} and K_m, respectively (cf. Fig. 4). This means that the initial rate measurement is not the only way to determine K_m and V_{max}/e, as long as the reaction obeys first order kinetics.

Effect of Cyanide—The interaction between the oxidized enzyme and cyanide was determined to be slow. For example, about 1 hour of preincubation of 0.5 to 1.0 μM cytochrome oxidase with 1 to 2 mM potassium cyanide at pH 7.0 and 0°C was necessary to attain an equilibrium between them. A mixture of 1 μM purified oxidase and 1 to 2 mM potassium cyanide could be brought to an equilibrium nearly instantly upon the addition of 2 to 5 μM ferrocytochrome c or a trace (about 0.1 mg per ml) of dithionite. Thus it seemed likely that a reduced form of the enzyme was a form of the enzyme involving complex formation with cyanide at a kinetically significant speed under the conditions of the present assay system, although both reduced and oxidized forms of the enzyme were known to form spectrally detectable complexes with the inhibitor (1, 22, 30). In fact, in 1952 Clauwes (31) had recognized in his study of mitochondrial respiration that the form of cytochrome oxidase that cyanide inhibited was present in large concentrations in the reduced system, in low concentrations in the steady state oxidized system, and in very low concentrations in the oxidized system. Under the present assay conditions, the enzyme-cyanide complex was found to dissociate fairly rapidly and to reach an equilibrium within 10 see after dilution (cf. Fig. 5). Since the amount of ferrocytochrome c oxidized during this pre-equilibrium period was negligible, initial rates were determined not at the time of the addition of the enzyme-cyanide complex (cf. Arrow A in Fig. 5), but at the time when the enzyme-cyanide complex reached a new equilibrium (cf. Arrow B in Fig. 5). In the presence of cyanide, the oxidation of ferrocytochrome c was no longer first order, so that initial rates were measured by the differential method only. Lineweaver-Burk plots of initial rates in the presence and absence of 5 to 10 μM cyanide are shown in Fig. 6. Cyanide appeared to be an inhibitor of the competitive type; this could be interpreted that the inhibitor combined with ES. The present kinetic analysis does not specify the nature of the ES complex. It could be one of the following: E_{ax-S}, E_{red-P}, or E_{red}.

Since dithionite brings a mixture of the oxidized enzyme and cyanide to an equilibrium and since the reduced form of the enzyme forms a spectrally distinct complex with cyanide in the absence of cytochrome c, there is a strong suggestion that...
the reduced form of the enzyme is the one with which cyanide complexes at a kinetically significant rate. This may represent the explanation of our observation of the uncompetitive inhibition of the enzyme by cyanide. $K_i$ values of cyanide were calculated from the intercepts at the $e/V_{\text{max}}$ axis and are given in Table II. $K_i$ values were relatively pH-insensitive at pH 6 to 8. It should be noted that the predominant form of cyanide in this pH range is HCN.

**Effect of Azide**—Although azide is known to be a potent inhibitor of cytochrome oxidase (32), there have been no reports indicating a spectrally distinct complex formed between them. When a 0 to 10 mM quantity of the enzyme was added to a reaction mixture containing 25 $\mu$M azide, it took 20 to 30 sec to establish an equilibrium between the enzyme and azide. Since during this period a significant amount of ferrocytochrome $c$ was oxidized, it was necessary to incubate the enzyme and azide together before the reaction was initiated. About 1 min of incubation of the oxidized enzyme and sodium azide was sufficient to establish an equilibrium. The dissociation of the enzyme-azide complex seemed very rapid as indicated by the fact that the oxidation of ferrocytochrome $c$ in the presence of azide obeyed first order kinetics. Lineweaver-Burk plots of initial rates in the presence and absence of azide (cf. Fig. 3) revealed that azide was an inhibitor which was noncompetitive with ferrocytochrome $c$ in agreement with Minnaert (8). $K_i$ values are given in Table II. In agreement with Keilin's original observation (32), $K_i$ values were extremely pH-sensitive: the azide inhibition was stronger at lower pH.

**DISCUSSION**

**Rate Equations**—Equation 5 was formulated on the basis of an experimental observation that ferrocytochrome $c$, one of the reaction products, was strictly a substrate-competitive inhibitor. Equation 5 predicts the usual hyperbolic relationship between initial rates and substrate concentrations as observed in the present study as well as in the previous works. Equation 6, an integrated form of Equation 5, also satisfactorily incorporated two important observations of Smith and Conrad (10); namely, the apparent first order time course of the reaction at all the cytochrome $c$ concentrations tested and the variation of the apparent first order rate constants as a function of the total concentration of cytochrome $c$ if $K_m = K_i$ was assumed. In fact, the present study clearly showed that the actual time course of the reaction did obey first order kinetics when $K_m = K_i$ and did not follow first order kinetics when $K_m \neq K_i$ (cf. Fig. 2 and Table I).

When the time course of the reaction obeys first order kinetics and thus $K_m = K_i$ is presumed, one can determine kinetic constants, $k_i = V_{\text{max}}/e$, $K_m$, and $k_o$ by measuring apparent first order rate constants at different concentrations of total cytochrome $c$. Since $K_m = K_i$ is a prerequisite condition, $K_i$ cannot be determined by this method. These kinetic constants were previously thought to be associated only with the initial rate measurement by the differential method, but the present method clearly shows that it is not the case. One of the important advantages of the measurement of apparent first order rate constants (24) is that the rate constants are dependent on the total cytochrome $c$ concentrations ($s_o + s_i$), but independent of the ratios of ferro- to ferrocytochrome $c$ ($s_i/s_o$). Thus it is not necessary to use 100% reduced cytochrome $c$ as a substrate in the integral method. A fully reduced cytochrome $c$ as a substrate is absolutely necessary for the initial rate measurement by the differential method. Although the apparent first order rate constant at a particular concentration of cytochrome $c$ was previously used to express the cytochrome oxidase activity (3, 10, 12-14, 24), it is more useful if the apparent first order rate constant is extrapolated to a maximal value at $(p_o + p_i) = 0$ according to the plots shown in
Let us now discuss the time course curve of the reaction measured at a fixed concentration of total cytochrome c (p_c + p = constant). If the effect of an inhibitory product is not considered, the order of the reaction with respect to s should change progressively from pseudo-zero to first order during the course of reaction as the degree of substrate saturation decreases depending on decreasing s:K_m ratios. The effect of an inhibitory product behaves in an opposite direction. As the reaction progresses and p increases from K_i >> p to p >> K_i, the product inhibition increases progressively as a function of p:s and p:K_i. Therefore, the actual time course of reaction results from a complicated combination of these two effects, as represented by Equation 6. If the product is a substrate-competitive inhibition and K_m = K_i, the situation is very much simplified, since Equation 6 becomes Equation 7. Equation 7 predicts that the time course of the reaction is apparently first order with respect to s. Thus, the observed first order time course of oxidation of ferrocytochrome c can be explained as a simple consequence of a combined effect of the progressively decreasing substrate saturation and the progressively increasing product inhibition at K_i - K_m. The observed time course curve, although it is apparently first order, does not necessarily indicate that the oxidation of ferrocytochrome c catalyzed by cytochrome oxidase is truly first order with respect to s at any s range or that K_m of ferrocytochrome c is infinitely large (33). The observed kinetics can be satisfactorily interpreted without assuming the formation of an inactive complex between cytochrome oxidase and ferrocytochrome c (10, 12, 18, 19, 33).

Recently McGuinness and Wainio (14) reported that truly initial rates determined by a differential method were zero order with respect to s which was varied from 10 to 50 μM. If initial rates were zero order with respect to s, their Lineweaver-Burk plot would be a straight line parallel to the 1/s axis, and thus the K_m value would have to be indefinitely small (K_m = 0). They, however, seemed to have obtained a definite value of about 10^{-5} M, which was calculated from their values of K_m:e = 10^6 and e = 10^{-5} M. They attributed the observed zero order kinetics to an assumption that the enzyme would be fully saturated at a high K_m:e ratio of 10^9. The degree of substrate saturation of enzyme cannot be determined by the K_m:e ratio. The determining factor is s:K_m. Under their experimental conditions, the s:K_m ratio ranged from 1 to 5. According to the present studies, these concentrations of ferrocytochrome c were definitely not enough to saturate the enzyme and thus zero order kinetics could not be expected under their conditions.

Spectrophotometric versus Manometric and Polarographic Assay Methods—As discussed previously, there are definite shortcomings in the manometric and polarographic assays of the cytochrome oxidase reaction in the presence of a reducing agent, because cytochrome c is not always fully reduced (2, 4, 8). Recently Smith and Camerio (34) described the polarographic assay of such a system under the conditions at which cytochrome c was more than 85% reduced. Aerobic steady states of cytochrome c in the system depend upon a number of factors such as pH, reactant concentrations, and the specific activity of the enzyme preparation (4), so that the conditions recommended by Smith and Camerio (34) are not always attainable, especially at low pH values and low cytochrome c concentrations. We believe that the spectrophotometric assay system established by Smith (24) and adapted in the present study is a preferable one to measure the reaction between cytochrome c and cytochrome oxidase. There is no doubt about the fact that the manometric and polarographic methods have valuable advantages to study the reaction among cytochrome oxidase, oxygen, and oxygen-competing inhibitors such as CO (5, 25, 35, 36).

Particle-bound versus Solubilized Enzymes—It has been well recognized that the cytochrome oxidase activity of the particulate preparation was greatly increased upon the addition of detergents which solubilize the enzyme from mitochondrial particles (4, 37-40). Lower k, values of the particulate preparation, a particle-bound enzyme, indicate that the interaction between ferrocytochrome c and cytochrome oxidase is definitely slower in the particle-bound enzyme than in solubilized enzymes (cf. Table 1). This slow interaction may have resulted in apparently smaller V_max/e values for the particulate preparation. Thus the particle-bound enzyme may not be saturated with ferrocytochrome c even at an infinite concentration of exogenous ferrocytochrome c. Such an assumption is supported by the recent observation of Smith and Camerio (37) that s/e values of a particle-bound cytochrome oxidase which was apparently saturated with exogenous ferrocytochrome c were greatly enhanced by further additions of other electron donors such as NADH.

Slater (33) noted that k_c values (k_c in his terminology) obtained with a particulate preparation varied greatly with the conditions. The interaction between exogenous ferrocytochrome c and the particle-bound enzyme or endogenous cytochrome c depends upon the physical state of the particle to which the enzyme or the endogenous cytochrome c is bound. Thus it is natural that k_c values of the particulate preparation are influenced by the factors which may affect the physical state of particles. In the case of solubilized enzymes such as the solubilized particulate preparation and the purified oxidase, k_c (or k_c) values are not significantly affected by such factors.

Apparent and True Second Order Rate Constants, k_1 and k_1 - A k_1 value of 10^8 M^{-1} sec^{-1} reported by Smith and Conrad (10) is in good agreement with k_c values found in the present study (see Table 1). Although V_max/K_m:e = k_1 has been assumed by previous workers (10, 33), this ratio or k_c is, in fact, k_1:K_2 (k_1 + k_2) as shown in Equation 10: k_c is equal to k_1 only if k_2 >> k_1. There are several methods to determine k_1 values (17). The most direct one may be the rate measurement by the flow method. If a k_1 value of 10^9 M^{-1} sec^{-1} is assumed, the half-time of the association of 1 μM ferrocytochrome c and 1 μM cytochrome oxidase is approximately 50 msec, which is certainly within the working range of flow methods. Recently Greenwood et al. (41, 42) have directly determined k_1 values of 4 × 10^9 to 4 × 10^10 M^{-1} sec^{-1} with purified preparations of cytochrome oxidase. Since these directly determined values of k_1 are approximately equal to the k_c values, we can circumstantially assume k_3 >> k_1 from Equation 10. Thus the k_c values in Table 1 may be treated as the true second order rate constant, k_c.
Binding Sites of Inhibitors on Cytochrome Oxidase (cf. Fig. 7)—Ferricytochrome c, a competitively inhibiting product, presumably interacts with the ferrocytochrome c-binding site of the enzyme. Although azide was thought to interact with cytochrome a3, there is no spectral evidence to support such an interaction. Steady state studies in the presence of the inhibitor (31), however, suggest that the reduction of cytochrome a3 is blocked by azide. At this time, no particular component of the enzyme can be assigned to the azide-binding site. Spectrophotometric evidence indicates that cyanide combines with both reduced and oxidized forms of cytochrome a3 (1, 22, 30). The conclusion of Keilin and Hartree (22) that the cyanide compound of reduced cytochrome a3 can be oxidized to the cyanide compound of oxidized cytochrome a3 by oxygen has been supported by a number of subsequent workers. Chance's observation (31) as well as the result presented here, however, strongly suggest that the rate of interaction between oxidized cytochrome a3 and cyanide seems to be too slow to account for this form of the cytochrome as the kinetically significant site of cyanide binding and that reduced cytochrome a3 is the enzyme site to which cyanide binds at a kinetically significant rate. Since cytochrome definitely blocks the reduction of cytochrome a3, we have to differentiate the site of cyanide action on the enzyme into cyanide-binding and cyanide-blocking sites as schematically shown in Fig. 7. Keilin and Hartree (22) noted the ability of cyanide to dissociate the reduced enzyme-CO complex. Gibson and Greenwood (43) published data indicating a competition between CO and cyanide for reduced cytochrome a3, while Warburg (35) could not observe any competition between cyanide and oxygen for cellular respiration. It remains to be proven whether the cyanide complex of reduced cytochrome a3 can be oxidized to its oxidized form by oxygen without releasing cyanide from the complex. Of course, one cannot exclude the possibility that the blocking of cytochrome a3 reduction may be caused by the interaction of cyanide with sites other than the heme prosthetic groups, for example, copper. The binding site of CO has been firmly established by numbers of investigators (1, 5, 22, 30, 35, 41, 42, 43-47).

SUMMARY

The aerobic oxidation of ferrocytochrome c catalyzed by cytochrome oxidase was kinetically analyzed in the presence and absence of inhibitors. Ferrocytochrome c is an inhibitor which is competitive with ferrocytochrome c. Its $K_i$ values are approximately equal to $K_m$ values of ferrocytochrome c at acidic and neutral pH values where the time course of the reaction apparently obeys first order kinetics with respect to the ferrocytochrome c concentration. At alkaline pH values, however, $K_i$ values are no longer equal to $K_m$ values and the time course of the reaction was found to deviate considerably from first order kinetics. A rate equation was formulated to represent the system under consideration. If $K_m = K_i$ was assumed, the equation satisfactorily represented the apparent first order kinetics of the time course of reaction and the variation of the apparent first order rate constant with changing the total cytochrome c concentration, as observed by Smith and Conrad. Thus it was concluded that the apparent first order time course of the reaction was a simple consequence of a progressive inhibition of the enzyme by a competitively inhibiting product, ferrocytochrome c. A new plotting method was introduced to determine several kinetic constants from apparent first order rate constants. Azide and cyanide were found to be non- and uncompetitive, respectively, with ferrocytochrome c. Possible sites of inhibitor binding on cytochrome oxidase were discussed.

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REFERENCES

35. Warburg, O., Heavy metal prosthetic groups and enzyme action, Oxford University Press, London, 1944.