The Reaction of Primate Cytochromes c with Cytochrome c Oxidase

ANALYSIS OF THE POLAROGRAPHIC ASSAY*

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The steady state reactions of a number of native and chemically modified primate and nonprimate cytochromes c with beef and rhesus monkey cytochrome c oxidases, monitored polarographically, were analyzed by studying 1) the presteady state kinetics of the high affinity reaction between the cytochromes c and purified beef cytochrome c oxidase; 2) the corresponding binding of the cytochromes c to the purified beef enzyme; 3) the presteady state reaction of N,N,N',N'-tetramethylphenylenediamine (TMPD), the reducing agent employed in the polarographic assay, with the various cytochromes c and their high affinity complexes with the purified beef enzyme; and 4) the presteady state low affinity reaction between ferrocytochrome c and the high affinity ferricytochrome c-beef cytochrome oxidase complex. It was concluded that the reduction of the enzyme-ferricytochrome c (EP) complex by TMPD is the rate-limiting step in the steady state polarographic assay, that the low maximal velocities for the high affinity reaction of simian cytochromes c with nonprimate cytochrome c oxidase result from a decreased rate of reduction of the EP complex by TMPD, and that this decrease may be due to evolutionary changes in the structures of the primate cytochromes c which result in the formation of too tight an EP complex. Finally, it was shown that the low affinity reaction of cytochrome c with beef cytochrome oxidase is dependent on whether a primate or a nonprimate cytochrome c is bound in the high affinity complex, demonstrating that the high and low affinity binding sites are interacting.

Cytochrome c is a small, basic, water-soluble protein of the mitochondrial respiratory chain that is located in the space between the inner and outer mitochondrial membranes, and functions in transporting electrons between cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV), (1, 2). It has recently been demonstrated that the surface area of cytochrome c which interacts with cytochrome oxidase or with cytochrome reductase is located on the front of the molecule, being centered around the point at which the positive end of the dipole axis crosses the surface of the protein near the P-carbon of phenylalanine 82 in horse cytochrome c (3–14). This interaction domain contains most, if not all, of the heme edge exposed on the front surface of the protein, the structure most likely involved in electron transfer. The electric field generated by the strongly asymmetric distribution of charged residues governs the correct alignment of the protein with its mitochondrial redox partners, accounting quantitatively for the changes in activity of many modified cytochromes c (14–16).

Recent studies (7, 13, 17–19) have shown that the kinetics of reaction of cytochrome c with cytochrome c oxidase measured polarographically, with ascorbate and TMPD as reducing agents, is biphasic with respect to the concentration of cytochrome c. Furthermore, the binding affinities measured directly with a variety of ferricytochromes c and purified preparations of cytochrome oxidase correspond to the Kₐ values observed for the high affinity kinetic phase (13, 17). While employing the steady state polarographic assay system (20–23) to study the reactions of a series of primate cytochromes c with primate and nonprimate cytochrome c oxidases, it was found that the proteins from prosimian species reacted identically to those from nonprimate mammals. Both prosimian and nonprimate cytochromes c reacted well with beef cytochrome c oxidase, but poorly with the rhesus monkey enzyme. In contrast, cytochromes c from simians, such as apes, and Old World and New World monkeys, exhibited high maximal velocities with the monkey oxidase, but very low maximal velocities with the beef enzyme, despite the formation of high affinity 1:1 complexes with the latter. To investigate this phenomenon, we studied 1) the steady state and the presteady state kinetics of reaction of a variety of native and chemically modified primate and nonprimate cytochromes c with submitochondrial particle preparations and with purified beef cytochrome c oxidase; 2) the presteady state reactions of TMPD, the reductant employed in the polarographic assay system, with the high affinity cytochrome c-cytochrome c oxidase complexes and with the free cytochromes c; 3) the

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The abbreviations used are: TMPD, N,N,N',N'-tetramethylphenylenediamine dihydrochloride; TNP, 2,4,6-trinitrophenol.
binding of the cytochromes c to purified beef oxidase; and 4) the presteady state reactions of horse and human ferrocytochromes c with the high affinity complexes formed between horse or human ferrocytochrome c and beef cytochrome c oxidase. It was concluded that the low maximal velocities for the reactions of the simian cytochromes c with the beef enzyme in the polarographic assay system result from a decrease in the rate of reduction of the enzyme product complex by TMPD, the step which was found to be rate-limiting. This does not appear to change the reactivity of TMPD with the free cytochromes c. In addition, the attenuated low affinity phase of the reaction of primate cytochrome c with beef cytochrome c oxidase appears to be due to the presence of a primate cytochrome c bound at the high affinity site, rather than to an intrinsic difference of the low affinity reaction of the primate and nonprimate cytochromes c for the low affinity kinetic phase.

Preliminary accounts of part of this work have appeared (24, 25).

EXPERIMENTAL PROCEDURES

Cytochromes c were prepared by the procedure of Margoliash and Walasek (26) as modified by Brautigan et al. (27). The human material was obtained at autopsy. Tissues from simian species, namely those from the Old World monkeys, Macaca mulatta (rhesus monkey) and Papio papio (baboon), and those from the New World monkeys, Ateles fusciceps (spider monkey) and Cebus apella (capuchin monkey), as well as those from the prosimian species, Nycticebus coucang (slow loris) and Tupaiia glis (tree shrew), were gifts from Dr. Morris Goodman and the Delta Regional Primate Center, Tulane University. TNP-lysine 13 horse and human cytochromes c were prepared by a modification (28) of the procedure of Wada and Okumura (29). Keilin-Hartree submicromolar particle preparations from beef and rhesus monkey heart muscle were prepared by the method of King (30) as modified by Ferguson-Miller et al. (17). Purified cytochrome c oxidase was prepared from beef heart (as described by Van Buuren (31). Tris, sucrose (grade I), Tween 20, and 2,6-dichlorophenolindophenol were obtained from Sigma and ascorbic acid and TMPD were from Eastman. All other chemicals were analytical reagent grade.

Separation of Polymeric Material—Prior to all characterizations, monomeric cytochrome c was separated from any polymeric material, as well as reductant (ascorbate) or oxidant (potassium ferricyanide), on columns (0.7 X 18 cm) of Sephadex G-50 superfine (Pharmacia) equilibrated in the appropriate buffers.

Reduction Potential Measurements—Reduction potentials were determined by a modification (13) of the optically transparent thin layer electrode method of Heineman et al. (32). Oxidative titrations were carried out at 23 °C in 150 mM acetate (Tris), pH 7.0, with 1.0 mM 2,6-dichlorophenolindophenol as mediator and were followed with a Cary model 17 recording spectrophotometer.

The Binding of Ferricytochrome c to Purified Beef Cytochrome c Oxidase—The high affinity binding between cytochrome c and cytochrome c oxidase was determined by a modification (13) of the gel filtration procedure of Hummel and Dreyer (33). Cytochrome c oxidase (2.0 nmol) was mixed with samples of cytochrome c (6 to 10 nmol) in a total volume of 100 pl and chromatographed at 23 °C on a column (0.7 X 28 cm) of Sephacryl S-200 superfine (Pharmacia) equilibrated in 25 mM acetate (Tris), pH 7.0, 25% Tween 20, pH 7.8, containing from 0.25 to 3.0 μM cytochrome c. The concentrations of cytochrome c and cytochrome c oxidase (cytochrome c oxidase) in the eluted complex, determined from the oxidized and reduced (dithionite) spectra (34), were corrected for the cytochrome c in the equilibration buffer and used to determine the proportion of cytochrome c to cytochrome c oxidase in the complex. The results of these studies were analyzed as described by Hughes and Klotz (35).

Steady State Reaction of Cytochrome c with Cytochrome c Oxidase—The reaction was followed polarographically with a Gilson model KM oxigraph and a Yellow Spring Instruments oxygen electrode in a thin Teflon sample cell. Ascorbate and TMPD (recrystallized from ethanol (36)) were employed as reductants (7, 17). Determinations were carried out at 25 °C in 25 mM acetate (Tris) pH 7.0, with cytochrome c concentrations ranging from 0.01 to 3.0 μM. The final concentrations of the beef and rhesus monkey Keilin-Hartree preparations in the assay media were 0.05 and 0.1 mg total protein/ml, respectively.

Rapid Kinetics—Rapid reactions were studied at 10 °C with a modified Perkin-Elmer spectrophotometer, equipped with a 2-cm optical path length reaction chamber, as previously described (37). The photomultiplier output signal was transferred via a log converter to a Datalab 905 transient recorder as a 1024 point data file and stored in a Hewlett-Packard 2100A computer. All traces were subjected to a five-point smoothing procedure. The rate constants were derived from the initial rate data using a least squares best fit (37). The experimental curves and those corresponding to the rate constants were displayed simultaneously to allow visual inspection of the fit.

The presteady state reaction of ferrocytochrome c with cytochrome c oxidase was monitored by following the reduction of the oxidase at 444 nm. It was found that a 3-fold excess of cytochrome c oxidase over cytochrome c sufficed to yield pseudo-first order kinetics of the high affinity reaction (37), such that the time courses studied accurately fit single exponentials. The reaction was studied over a buffer concentration range of 500 to 133 mM acetate (Tris, pH 7.8), and 0.8 to 3.0 μM cytochrome c oxidase, pH 7.8, the lowest ionic strength at which the reaction was slow enough to be accurately followed. The concentration of cytochrome c was 0.8 μM while that of the oxidase ranged from 3 to 10 μM. The logarithms of the observed second order rate constants were plotted as a function of the square root of the ionic strength. The plot was extrapolated to lower ionic strength to obtain an approximation of the rate constant at 25 mM acetate (Tris).

The oxidation of ferrocytochrome c by the high affinity ferricytochrome c-beef oxidase complex, monitored at 444 nm, was examined in 25 mM cacodylate (Tris), pH 7.8, containing 0.25% Tween 20. The concentration of ferricytochrome c was 1 μM, and the concentration of the ferricytochrome c-beef oxidase complex was varied from 1.6 to 4.1 μM for the horse ferricytochrome c-oxidase complex, and from 3.2 to 7.5 μM for the human ferricytochrome c-oxidase complex. The high affinity complexes of horse and human ferricytochrome c with beef oxidase were prepared by mixing at 1:1 molar excess of cytochrome c with cytochrome oxidase in 25 mM cacodylate (Tris), pH 7.8, buffer containing 0.25% Tween 20. Assuming a dissociation constant of 20 nM for the high affinity complexes, less than 1% of complex will be dissociated under the conditions employed (13). Since an excess of ferricytochrome c-oxidase complex was used in all cases, the small proportion of free ferricytochrome c did not significantly interfere with the low affinity reaction of ferrocytochrome c.

The reaction of TMPD with ferrocytochrome c, monitored by following the reduction of cytochrome c at 416 nm, was examined in 25 mM acetate (Tris), 0.25% Tween 20, pH 7.8, at 0.8 μM cytochrome c. The reaction of cytochrome c-oxidase complex was varied from 1.6 to 100 μM. The reaction of the cytochrome c-oxidase complex by TMPD was studied under the same conditions, except for the addition of 3 μM cytochrome c oxidase to ensure full complexing of the cytochrome c. In these experiments, the reduction of cytochrome c was followed at 444 nm, namely by monitoring the cytochrome oxidase. Since the rate of reduction of the enzyme by ferrocytochrome c was found to be 3 to 6 orders of magnitude faster than that of the cytochrome c-oxidase complex by TMPD under the same conditions, the rate observed was that for the reduction of bound cytochrome c by the dye. The TMPD concentration was kept fully reduced by the addition of a 4-fold molar excess of ascorbate, the same excess employed in the steady state cytochrome c-oxidase polargraphic assay. During the time course examined, the reaction between the reducing agents employed and the enzyme was found to be negligible.

The standard deviation for all the stopped flow data was approximately 5%.

RESULTS

The Steady State Reaction between Cytochrome c and Cytochrome c Oxidase—The maximal velocity for the high affinity phase of the reaction (Vmax) of human cytochrome c with rhesus cytochrome oxidase is twice as large as that for horse cytochrome c (Fig. 1). In contrast, the Vmax for the reaction of the human protein with beef cytochrome c oxidase is less than 15% that for horse cytochrome c (Fig. 2), as determined by the polargraphic assay system (7, 17). This
In an effort to determine the reason for the low maximal velocity of the reaction of the human protein with beef oxidase, the kinetics of reaction of TNP-lysine 13 horse and TNP-lysine 13 human cytochromes c with the beef and rhesus oxidases were examined (Figs. 1 and 2; Table I). Modification of the horse protein decreased its activity with both enzyme systems. This was expected, as trinitrophenylation neutralizes the positive charge of lysyl residue 13 near the center of the oxidase interaction domain on cytochrome c (3-5, 7, 8, 13, 22, 29), greatly decreasing the binding affinity for the enzyme (see Fig. 3). Similarly, the $K_m$ for the reaction of TNP-lysine 13 human cytochrome c with rhesus monkey cytochrome oxidase was increased as compared to that for the unmodified human protein (Fig. 1). However, the $V_{max}$ for TNP-lysine 13 human cytochrome c reacting with the beef enzyme was four times higher than that for native human cytochrome c, and 50% of the value observed for the reaction of native horse cytochrome c (Fig. 2). It is remarkable that notwithstanding the loss of a positive charge involved in the interaction between cytochrome c and cytochrome c oxidase, and the interposition of a bulky aromatic group on the front surface of the molecule, this modification of human cytochrome c resulted in an increased activity with beef oxidase despite a decreased binding affinity (see Fig. 3). Clearly, some change in either the cytochrome c or in the enzyme-substrate complex must overshadow the unfavorable effects of the modification.

Reconstruction Potentials—The reduction potentials of cytochromes c from representative ape, Old World monkey, New World monkey, prosimian, and nonprimate mammalian species are listed in Table I. The $E^0$ values calculated from Nernst plots ($n = 0.8$ to 1) were quite similar and ranged from 268 to 276 mV. As these values may be considered to be the same within experimental error, the above kinetic differences are unlikely to be related to the thermodynamic control of the enzymic reaction.

The Binding of Cytochrome c to Cytochrome c Oxidase—The high affinity binding of several native and singly trinitrophénylated cytochromes c to purified beef cytochrome c oxidase are presented in Fig. 3. In all cases, an approximately 1:1 high affinity complex was observed, indicating that the low maximal velocities of the reactions of the simian proteins with the beef enzyme are not due to a lack of binding. At higher cytochrome c concentrations (>1 μM for the horse protein), molar ratios of cytochrome c to cytochrome aa3 greater than 1:1 were apparent, confirming the presence of additional lower affinity binding sites(s) (7, 8, 13, 17). The dissociation constant, $K_d$, for the high affinity binding of horse cytochrome c was found to be approximately $4 \times 10^{-8}$ M (Table I). It is apparent from Fig. 3 that both the human and spider monkey proteins bind to the beef enzyme at least as well as does horse cytochrome c.

As shown in Table I, the $K_d$ values obtained for all of these high affinity complexes are essentially the same as the $K_m$ values obtained for the reactions of these cytochromes c with beef cytochrome c oxidase. Similar results have been obtained by Osheroff et al. (13), who found that the $K_d$ and the $K_m$ values were the same for native horse cytochrome c and a series of ten different singly modified 4-carboxy-2,6-dinitrophenyllysine cytochromes c. This is in accord with the original suggestion of Ferguson-Miller et al. (7, 17) that the $K_m$ of this reaction, as monitored by the polarographic assay system, is the dissociation constant of the cytochrome c-cytochrome c oxidase complex.

The Presteady State Reaction between Cytochrome c and Cytochrome c Oxidase—The overall kinetic scheme for the polarographic assay system is shown in Fig. 4. As earlier demonstrated (7, 16) and confirmed below, in the polarographic assay system 2)

N. Osheroff and E. Margoliash, unpublished results.
The Cytochrome c-Cytochrome c Oxidase Reaction

Some high affinity phase kinetic parameters and physical constants for several cytochromes c

| Cytochrome c | Beef oxidase | Rhesus monkey oxidase | Enzyme-bound cytochrome c | Free cytochrome c | Physical properties |
|--------------|--------------|-----------------------|--------------------------|------------------|--------------------|
|              |  \( K_{m}^{a} \) | \( V_{max}^{a} \) | \( k_{\text{forward}}^{a} \) | \( E'_{c} \) | \( pK_{a} \) |
| Horse        | 3 x 10^{-8}  | 3 x 10^{-8}          | 53.5 \( M^{-1} s^{-1} \) | 2.2 x 10^{9} | 273 \( \text{mV} \) |
| Slow loris   | 3 x 10^{-8}  | 3 x 10^{-8}          | 53.5 \( M^{-1} s^{-1} \) | 2.4 x 10^{9} | 269 \( \text{mV} \) |
| Spider monkey| 1 x 10^{-8}  | 1 x 10^{-8}          | 14.5 \( M^{-1} s^{-1} \) | 1.0 x 10^{10} | 272 \( \text{mV} \) |
| Human        | 1 x 10^{-8}  | 1 x 10^{-8}          | 8.0 \( M^{-1} s^{-1} \)  | 1.5 x 10^{10} | 274 \( \text{mV} \) |
| TNP-Lys-13 horse | 4 x 10^{-7} | 2 x 10^{-7}          | 27.0 \( M^{-1} s^{-1} \) | 1.5 x 10^{10} | 272 \( \text{mV} \) |
| TNP-Lys-13 human | 5 x 10^{-8} | 3 x 10^{-7}          | 31.0 \( M^{-1} s^{-1} \) | 1.6 x 10^{10} | 268 \( \text{mV} \) |

\( a \) Determined at 25 °C, employing purified beef cytochrome oxidase.

\( b \) Values were obtained at 25 °C employing Keilin-Hartree particle preparations.

\( c \) Determined at 10 °C, employing purified beef cytochrome oxidase.

\( d \) Determined at 10 °C.

\( e \) Determined in 150 mM acetate (Tris), pH 7.0 at 25 °C. E', values of 275, 272, 273, and 275 mV were also found for beef, tree shrew (prosimian), capuchin monkey (New World monkey), and baboon (Old World monkey), respectively.

\( f \) Determined in 10 mM acetate (Tris); from Ref. 23.

\( g \) As the reaction was monophasic and of high \( K_{m} \), the range of values given extends from one-half to the \( V_{max} \) actually observed.

Graphical assay system TMPD reduces the cytochromes c-cytochrome oxidase complex directly, and since the low maximal velocities exhibited by the simian cytochromes c reacting with beef cytochrome oxidase do not result from either a lack of binding or a change of reduction potential, they must be caused by a change in one or both of the following individual kinetics steps of the reaction. A block in the reaction can occur before the formation of \( EP \) by a decrease in \( k_{a} \), the rate of electron transfer between ferrocytochrome c and the enzyme, or after the formation of \( EP \) by a decrease in \( k_{TMPO} \), the rate of reduction of \( EP \) to \( ES \) by TMPD.

To test the first possibility, the presteady state reaction of several ferrocytochromes c with purified beef cytochrome oxidase were followed by stopped flow. The results are given in Fig. 5 in which the pseudo-first order rate constants are plotted against the concentration of the component present in excess. An excess of cytochrome aa3 was employed to ensure that only the high affinity reaction was monitored (37). Assuming that the rate limiting step in the forward reaction (\( k_{\text{forward}} \)) through the formation of \( EP \) is \( k_{1} \), the rate constant for the association of the ES complex, \( k_{a} \), the slope of the lines in Fig. 5 are the second order rate constants for the forward reactions. Similarly, the intercepts with the ordinate are \( k_{2} \), the first order rate constants for the dissociation of ES to E and S, and the intercepts with the abscissa are the dissociation constants of ES, namely \( k_{-a}/k_{1} \).

The experiments shown in Fig. 5 were carried out in 133 mM acetate (Tris), pH 7.8, since at lower ionic strengths the rates of some of the reactions were too fast to measure accurately in the stopped flow apparatus. The fastest forward rate constant (\( k_{\text{forward}} \)) was observed for human cytochrome c, followed by spider monkey, slow loris, horse, TNP-lysine 13 human, and TNP-lysine 13 horse cytochromes c, in that order.

A study of the ionic strength dependence of this reaction was

\[
E + S \xrightleftharpoons[k_{-a}]{k_{a}} ES \xrightarrow[k_{2}]{k_{1}} EP \xrightarrow[k_{TMPO}]{k_{TMPO}} E + P
\]

\( ^{a} \) For this to be the case, the rate of the reaction \( E + S \xrightarrow[k_{-a}]{k_{a}} ES \) must be considerably slower than that of \( ES \xrightarrow[k_{2}]{k_{1}} EP \). Two lines of evidence support this contention. First, there is a strong correlation between \( k_{\text{forward}} \), \( K_{m} \), and \( K_{a} \) (Table I). Second, when the rates of electron transfer (\( k_{1} \)) between either human or horse cytochrome c and beef oxidase in a preformed complex were determined directly in low temperature single turnover experiments, they were found to be identical (38). This is in contrast to the 2.5-fold difference found for the forward rate constants of these two cytochromes c in the present study (Table I), once again indicating that \( k_{\text{forward}} \) reflects \( k_{1} \), not \( k_{2} \).
carried out over a range of buffer concentrations up to 500 mM. A linear relationship between the logarithm of the second order rate constants and the square root of the ionic strength was observed (not shown), the native proteins exhibiting slopes of $-9.2$ to $-9.4$ and the two derivatized proteins yielding distinctly smaller slopes, $-6.5$ for the TNP-horse protein and $-6.8$ for the TNP-human cytochrome $c$. The forward rate constants for the reactions were estimated by extrapolation to an ionic strength of $25\text{mM}$, and are listed in Table I. The values obtained for this ionic strength were $35$ to $185$ times those in the $133\text{mM}$ buffer. Such numbers can only be considered approximations, since there is no certainty that the linearity of the relation is maintained over the ionic strength range of the extrapolation. However, any deviation is likely to be toward higher rather than lower $k_1$ values (39), so that the forward rate constants listed in Table I can be taken to be approximations of their minimal values.

From the above presteady state results on the oxidation of ferrocytochrome $c$ by beef cytochrome oxidase, it is very unlikely that either $k_1$ or $k_2$ are rate limiting in the polarographic assay. Moreover, differences in $k_{\text{forward}}$ cannot explain the differences in activity observed between the simian cytochromes $c$, as compared to the prosimian and nonprimate proteins, as had previously been proposed (24, 40). Indeed, the higher primate proteins exhibited the fastest $k_1$ values, as determined by measuring the rates of reaction from free cytochrome $c$ through electron transfer to the oxidase ($k_{\text{forward}}$). An even more dramatic example of the lack of correlation between polarographic $V_{\text{max}}$ values and the forward rate constants is provided by the TNP-lysine $13$ human cytochrome $c$. This modified protein has a steady state $V_{\text{max}}$ value four times that of native human cytochrome $c$ in $25\text{mM}$ acetate (Tris), pH $7.8$ (Fig. 2; Table I), while under the same presteady state conditions, the forward rate constant is decreased by a factor of $100$ (Table I).

**The Presteady State Reaction between TMPD and the Ferricytochrome c-Cytochrome c Oxidase Complex**—To determine whether the rate limiting step in the reaction of the simian proteins with beef cytochrome oxidase occurs at a step following the formation of EP, the presteady state reduction of preformed ferricytochrome $c$-beef cytochrome $c$ oxidase complexes by TMPD was monitored by stopped flow. The results, presented in Fig. 6 and Table I, show that the relative magnitudes of the rate constants for this reduction correlate closely with the polarographic $V_{\text{max}}$ values observed for the reaction of these cytochromes $c$ with beef oxidase (Fig. 3 and Table I). The complexes of the beef enzyme with horse and slow loris cytochromes $c$ are the most active with TMPD, yielding very similar rate constants, and are the most active polarographically, having identical $V_{\text{max}}$ values. The complex of beef cytochrome oxidase with spider monkey cytochrome $c$ is much less active in both assay systems, while the complex with the human protein exhibits even less activity. This correlation also extends to the TNP-modified cytochromes $c$. The TNP-lysine $13$ horse cytochrome $c$-beef cytochrome oxidase complex is less readily reduced by TMPD than the complex with the native protein, just as its $V_{\text{max}}$ is lower than that for horse cytochrome $c$. In contrast, the same modification of human cytochrome $c$ doubles the rate constants for the reduction by TMPD and quadruples $V_{\text{max}}$. The expected opposite pattern of reactivities of TMPD with the various cytochromes $c$ bound to rhesus cytochrome oxidase could not be examined as too little tissue was available for the preparation of sufficient purified enzyme.

These correlations strongly suggest that the rate-limiting step in the reaction of the simian cytochromes $c$ with beef cytochrome $c$ oxidase in the polarographic assay system results from a decrease in $k_{\text{TMPD}}$, the rate of reduction of EP by TMPD (Fig. 4). As discussed below, this reduction appears to be rate limiting in all cases (Table I).

**The Presteady State Reaction between TMPD and Cytochrome c**—Since the complexes of the various cytochromes $c$...
with beef cytochrome c oxidase show large differences in their activities with TMPD, the question arises whether this phenomenon is a property of the cytochromes c themselves, or of the complex. This was examined by following the presteady state kinetics of the reduction of these cytochromes c by the dye. The results are given in Table I and show that over the entire range of proteins the second order rate constants are essentially the same. Clearly, the variations in the rates of reduction of the EP complexes by TMPD are due to differences in the cytochromes c which affect the structure of the complex, but are not reflected in the reduction of the free cytochromes c.

The Presteady State Reaction between Ferrocytochrome c and the Ferricytochrome c-Cytochrome c Oxidase Complex—In addition to yielding a decreased $V_{max}$, the simian cytochromes c exhibit an attenuated second phase with an increased $K_m$, as compared to the values observed for the reaction of horse cytochrome c with beef cytochrome oxidase. To assess the nature of the low affinity reaction of human cytochrome c with the beef enzyme, the presteady state reaction of both horse and human cytochromes c with the high affinity human cytochrome c-beef cytochrome oxidase complex and also with the horse cytochrome c-beef cytochrome oxidase complex was examined under low ionic strength conditions (25 mM cacyclolate (Tris), pH 7.8) (Fig. 7).

It has previously been shown for the presteady state reaction of horse ferrocytochrome c with beef cytochrome oxidase that there are two kinetically distinct reactions (37). The slower reaction is not due to a slow dissociation of the initially formed complex, followed by a rapid reaction of a second molecule of ferrocytochrome c, since it is dependent on the free ferrocytochrome c concentration. Moreover, the observed pseudo-first order rate constants for this process ranged from 1 to 2 orders of magnitude larger than the first order rate constant for the dissociation of the cytochrome c-cytochrome oxidase high affinity complex of approximately 5 electrons/s, as estimated from a variety of experiments (19, 41, 42).

As shown in Fig. 7, a second order rate constant of approximately $3.5 \times 10^3$ M$^{-1}$ s$^{-1}$ was determined for the reaction of horse ferricytochrome c with the horse ferricytochrome c-beef cytochrome oxidase complex. In addition, the reaction between human ferrocytochrome c and the horse ferricytochrome c-beef oxidase complex was indistinguishable from the reaction of horse cytochrome c with this complex. This suggests that the low affinity interaction of horse or human cytochrome c with the beef enzyme, in the presence of horse cytochrome c bound to the high affinity site, is the same. However, the reaction of human and horse cytochromes c with the human cytochrome c-beef cytochrome oxidase complex yielded second order rate constants of $1 \times 10^3$ M$^{-1}$ s$^{-1}$ and $7.4 \times 10^3$ M$^{-1}$ s$^{-1}$, respectively. This decrease in $k_{forward}$ for the reaction of a second molecule of cytochrome c with the human cytochrome c-beef cytochrome oxidase complex, as compared to the reaction with the horse cytochrome c-beef cytochrome oxidase complex, is the first direct evidence for interaction between the high and low affinity sites on beef cytochrome oxidase, and is consistent with the observed increase in $K_m$ for the reaction of the simian cytochromes c with this oxidase.

DISCUSSION

A strong correlation has been found between the low maximal velocities observed polarographically for the high affinity reaction ($V_{max}$) of simian cytochromes c with beef cytochrome c oxidase, and the decreased rate constant for the reaction of the enzyme-product complex by TMPD. Furthermore, there is no correlation between the $V_{max}$ values for the reaction of these cytochromes c with beef cytochrome oxidase and their reduction potentials, high affinity binding to the enzymes, presteady state $k_{forward}$ values, or the rates of reduction of the free cytochromes c by TMPD.

For the low $V_{max}$ values to result from a decreased $k_{forward}$, the rate of reduction of the EP complex by TMPD at saturating substrate concentrations must be the rate-limiting step in the polarographic assay. Assuming that the oxidase-cytochrome c complex is entirely in the EP form, the condition which will maximize the rate of reduction of the system by TMPD, and that the concentration of TMPD is that employed in the polarographic assay (0.7 mM), then at $V_{max}$, utilizing the values for $k_{forward}$ and $k_{forward}$ given in Table I, one can estimate the expected maximal turnover number for the enzyme. The concentration of substrate required to saturate the high affinity reaction was taken to be about 2 orders of magnitude above the $K_m$ representing 99% saturation. For the reactions with horse, slow loris, spider monkey, and human cytochromes c, the maximal turnover numbers would be greater than $10^5$ electrons/s if $k_{forward}$ is rate limiting for all these cytochromes c, while for the TNP-lysine 13 horse and human cytochromes c, these values would be greater than $5 \times 10^5$ electrons/s. However, if $k_{forward}$ is rate limiting, the expected maximal turnover numbers would vary from 7 to 17 electrons/s for the different native and modified cytochromes c listed. The latter values are in excellent agreement with the maximal turnover numbers observed with purified preparations of beef cytochrome oxidase in the polarographic assay, such as the 17.5 electrons/s reported by Smith et al. (19) for beef cytochrome c, and 10 electrons/s reported by Rosevear et al. (42) for horse cytochrome c. When Kelin-Hartree mitochondrial particle preparations are used as the source of cytochrome oxidase in the polarographic assay, the observed maximal turnover numbers are up to 1 order of magnitude higher than for purified preparations of cytochrome oxidase (7, 16), still much slower than the expected maximal turnover numbers estimated for a
rate-limiting $k_{\text{forward}}$. Indeed, $k_{\text{forward}}$ with the particle preparation is not likely to decrease significantly from that measured with the purified oxidase, since the $K_a$ values for the high affinity phase are about the same with both types of preparation (17, 19). Thus, clearly the TMPD reduction of the $E'P$ complex is rate limiting at saturating substrate concentrations in the reaction of the cytochromes $c$ studied here with beef cytochrome oxidase.

As has been previously found, the $K_a$ values for the reactions of the unmodified as well as for the derivatized cytochromes $c$ with beef oxidase appear to accurately reflect the $K_a$ values for their binding to the enzyme (3, 13, 17). Therefore, the polarographic oxidase assay is the system of choice for examining and describing kinetic differences which are due to changes in binding. On the other hand, the $V_{\text{max}}$ for the reaction, as monitored polarographically, is best expressed by [total oxidase] $[\text{TMPD}] k_{\text{TMPD}}$, a term which is obviously nonphysiological. Even though no direct functional significance can be attached to this $V_{\text{max}}$, it is clear that, for the primate cytochromes $c$, the decreased $V_{\text{max}}$ values are indicative of changes in the cytochrome $c$-cytochrome $c$ oxidase complex, which result in making the enzyme-bound cytochrome $c$ less accessible to TMPD. The reduction of the ferricytochrome $c$-cytochrome oxidase complex by TMPD appears to be sensitive to the nature of the components of the complex, as well as to the environment of the oxidase. This is exemplified by the differences noted above between simian and nonsimian cytochrome $c$ bound to beef oxidase, and also those observed between purified and mitochondrial membrane-bound oxidases.

The properties which lead to altered $E'P$ complexes between the simian proteins and beef oxidase are not known. However, a straightforward correlation exists between $V_{\text{max}}$, $k_{\text{TMPD}}$, and $K_a$. The simian proteins bind to beef oxidase considerably more tightly than does the horse protein (Table 1). With such a tight binding, it is likely that TMPD cannot easily enter the complex. Conversely, trinitrophenylation of lysine 13 in human cytochrome $c$ increases the $K_D$ value to approximately that of the horse protein and concomitantly increases its $V_{\text{max}}$, by a factor of 4. This modification of a lysyl residue with the enzymatic domain on the molecular surface of cytochrome $c$ (3-14) interferes with the binding to the enzyme (Table 1) and presumably facilitates the access of TMPD to the enzyme-bound cytochrome $c$. A significant aspect of these correlations is that tightness of binding is clearly a property of the cytochrome $c$-cytochrome oxidase complex and not of the free cytochrome $c$, since the rates of reduction by TMPD of all the free cytochromes $c$ tested were approximately the same.

As was shown to be the case for the high affinity reaction, the decrease in maximal velocities observed for the low affinity phase of the reaction of simian cytochromes $c$ with beef oxidase may arise from a decreased rate of reduction of the enzyme-product complex, now containing two molecules of product ($EPP$). A direct demonstration was not attempted with the presteady state experiments employed, because in addition to the technical difficulties attending the extremely high enzyme and ferricytochrome $c$ concentrations required, it is unlikely that the reactions of TMPD with the high affinity and the low affinity bound cytochromes $c$ can be readily distinguished. Alternatively, $V_{\text{max}}$, may be determined by some other rate-limiting process. However, the increase in $K_a$ may be explained, at least in part, by the decrease in $k_{\text{forward}}$ for the reaction of a second molecule of cytochrome $c$ with the human ferricytochrome $c$-beef oxidase complex, as compared to that with the horse cytochrome $c$-beef oxidase complex. The fact that the differences in activity of either simian or horse cytochrome $c$ at the low affinity site depends upon whether the high affinity site is occupied by a simian or by horse cytochrome $c$ implies interaction between the two sites and limits possible interpretation of the low affinity phase of the reaction between cytochrome $c$ and cytochrome oxidase.

It should not be overlooked that the proposed model for the decreased activity of simian cytochromes $c$ with beef cytochrome oxidase does not of itself explain why horse cytochrome $c$ exhibits lower $V_{\text{max}}$ values than the simian cytochromes $c$ in the reaction with rhesus monkey oxidase. As purified cytochrome oxidase from a higher primate species was not available, presteady state kinetics and binding data were not obtained, precluding an adequate analysis of the corresponding reactions. From the limited information provided by the steady state kinetics, it would appear that parameters other than the high affinity binding may be involved.

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