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Studies of the Kinetics of Oxidation of Cytochrome *c* by Cytochrome *c* Oxidase: Comparison of Spectrophotometric and Polarographic Assays†

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ABSTRACT: The kinetics of oxidation of cytochrome *c* by cytochrome *c* oxidase were studied by spectrophotometric assays of the oxidation of soluble ferrocytochrome *c* and by polarographic measurements of O_2 uptake in the presence of *N,N,N′,N′*-tetramethylphenylenediamine plus ascorbate. Kinetic measurements by the two methods were compared by using different kinds of cytochrome *c* oxidase preparations and varying experimental conditions with cytochrome *c* concentrations between 0.05 and 5 μM. With both the spectrophotometric and the polarographic methods the most rapid rates per cytochrome *c* concentration were found at concentrations of cytochrome *c* around 0.1 to 0.25 μM. However, the two methods showed very different responses to changes of experimental conditions. Under some conditions the rates of O_2 uptake measured by the polarographic method were the same as the rates calculated from the spectrophotometric measurements of cytochrome *c* oxidation; under other conditions the measured rates of O_2 uptake were as much as 30-fold greater than the calculated rates. "Apparent K_M" values derived from plots of v/S against v are different when derived from data from the two methods under some experimental conditions. The rates of O_2 uptake in the presence of *N,N,N′,N′*-tetramethylphenylenediamine, ascorbate, and increasing concentrations of cytochrome *c* correlate with the concentrations of cytochrome *c*, which remain oxidized in the aerobic state under these conditions. The data suggest that some conditions promote the formation of an especially reactive cytochrome *c*–cytochrome oxidase complex.

Two different kinds of methodology have been utilized in recent studies of the kinetics of cytochrome *c* oxidase (EC 1.9.3.1) using different species or derivatives of cytochrome *c*, with the aim of gaining insight into its mechanism of action (Staudenmayer et al., 1976; Smith et al., 1976, 1977; Errede & Kamen, 1978; Ferguson-Miller et al., 1976, 1978a).

(1) First is the oxidation of pure soluble cytochrome *c* by the oxidase, followed spectrophotometrically. The reaction is first order in ferrocytochrome *c* with concentrations of cytochrome *c* between 0.04 and 100 μM in buffers such as 0.05 M Tris[1]–acetate, Tris–maleate, or phosphate or 0.1 M Mes or phosphate at pH values between 6 and 7.4 (Smith & Conrad, 1956; Smith et al., 1973; Errede & Kamen, 1978). However, the first-order rate constant decreases with increasing concentration of cytochrome *c* in the reaction mixture, but not in a linear fashion. The observed kinetics have been interpreted as reflecting rates of association and dissociation of cytochrome *c* and the oxidase (Minnaert, 1961; Yonetani & Ray, 1965; Errede & Kamen, 1978). Most recently Errede & Kamen (1978) obtained data over a wide range of concentrations of cytochrome *c* and could explain the observed kinetics by assuming the binding of two molecules of cytochrome *c* with different affinities for the oxidase.

(2) Second is the polarographic measurement of O_2 uptake in the presence of a reductant of cytochrome *c*. Ascorbate has often been utilized as the reductant, where the rate of O_2 uptake divided by four was found to equal the rate of oxidation of cytochrome *c* followed spectrophotometrically when the cytochrome *c* was kept nearly completely reduced during the reaction; rather high concentrations of ascorbate are required (Smith & Camerino, 1963a). Ferguson-Miller et al. (1976) employed a combination of TMPD plus a lower concentration of ascorbate and reported that the cytochrome *c* remained

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[1] Abbreviations used: SMP, submitochondrial particles; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine dihydrochloride; DOC, sodium deoxycholate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris-(hydroxymethyl)aminomethane.

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more than 98% reduced under their experimental conditions. TMPD can reduce the endogenous cytochrome *c* of mitochondrial membrane vesicles, while ascorbate cannot (Mochan & Nicholls, 1972). Plots of the data of Ferguson-Miller et al. (1976, 1978a) as v/S against v (Eadie–Hofstee plots) gave two linear parts; calculations of "apparent K_M" from the slopes of these lines were interpreted as evidence of binding constants of two molecules of cytochrome *c* to the oxidase, one with "high affinity" and the other with "low affinity". These binding constants were different from those calculated by Errede & Kamen from their spectrophotometric assays made under different experimental conditions.

We have been impressed with what appear to be interesting differences in the two kinds of assay methods. There have been no comparisons of oxidase activities measured by the two procedures under the same experimental conditions with very low concentrations of cytochrome *c* (up to 0.7 μM). Thus, we have made such studies with a number of different kinds of oxidase preparations and varying cytochrome *c* concentrations between 0.05 and 5 μM. With both methods the highest relative activities were found with cytochrome *c* concentrations between 0.05 and 0.25 μM, irrespective of the cytochrome oxidase content. However, variations of pH, buffer type, or ionic strength affected the two methods in different ways, so that while under some conditions the O_2 uptake rates measured polarographically in the presence of TMPD plus ascorbate were similar to the corresponding rates of oxidation of cytochrome *c* measured spectrophotometrically, under other conditions the measured rates of O_2 uptake were as much as 30-fold greater. The O_2 uptake rates were found to correlate with the concentration of cytochrome *c* remaining *oxidized* in the aerobic state in the presence of TMPD plus ascorbate.

We would like to report some of the experience of the last few years in working with the two methods and to discuss the implications such observations may have for the mechanism of the reaction of cytochrome *c* with cytochrome *c* oxidase. Preliminary presentations of some of these data have been made (Smith et al., 1978a,b).

Materials and Methods

Preparations. Most of the experiments reported here were made with submitochondrial particles (SMP) prepared from beef heart mitochondria by the method of Lee & Ernster (1967). Usually the cytochrome *c* was first removed from the frozen and thawed mitochondria by stirring them with cold 0.075 M phosphate buffer, pH 7.5, by using 1 mL of buffer for each 30 mg of protein and then collecting the washed mitochondria by centrifugation. The suspension was acidified to pH 5 with acetic acid, stirred for 15 min, and centrifuged, and the pellet was suspended in cold 0.5 M sucrose containing 0.01 M phosphate buffer, pH 7.0. The pH was brought to near 7 by addition of 0.2 M Na_2HPO_4, and then the treated mitochondria were collected by centrifugation and suspended in cold 0.25 M sucrose containing 0.02 M phosphate buffer, pH 7.4. The SMP were stored at −20 °C.

When the SMP were treated with detergent, 10% sodium deoxycholate (DOC) or Triton X-100 was added to a concentrated suspension (at least 14 mg of protein per mL) to make a mixture of 1 mg of detergent per mg of protein; then the mixture was diluted to the desired extent with cold distilled water (Smith & Camerino, 1963b).

Cytochrome *c* oxidase was purified from beef heart by the method of Hartzell & Beinert (1974). Cytochrome *c* was isolated from beef heart according to Margoliash & Walasek (1967) and further purified by isoelectric focusing. For use in the spectrophotometric assays, the cytochrome *c* was reduced with $NaBH_4$ (Smith et al., 1974). The concentration of cytochrome *c* in solution was calculated from the absorbance at 550 nm of the reduced pigment by using 27.6 as the millimolar extinction coefficient (Margoliash & Frohwirt, 1959).

The content of cytochrome aa_3 in the oxidase preparations was measured following the method of Vanneste (1966) and the content of cytochrome *c* by the method of Williams (1968).

Chemicals. Sodium ascorbate, cholic acid, TMPD, and Triton X-100 were all obtained from Sigma Chemical Co. The sodium ascorbate and cholic acid were recrystallized from hot water and from ethanol, respectively, and the TMPD was brought to pH 6 with NaOH.

Assay Methods. Cytochrome *c* oxidase was assayed polarographically with a Clark O_2 electrode at 26 °C in a water-jacketed chamber containing 2.6 mL (Smith & Camerino, 1963a); rates are expressed as micromolar O_2 uptake per second. The contents of the reaction chamber are given with the figures and tables. Oxidase activity was measured spectrophotometrically with an Aminco DW2a dual wavelength spectrophotometer at 418 or 550 nm in the split beam mode following the method of Smith & Conrad; first-order rate constants were calculated (Smith & Conrad, 1956). Velocities of reaction were calculated from the product of the rate constants and the concentrations of ferrocytochrome *c*. These were divided by four to obtain the equivalent rates of O_2 uptake. "Apparent K_M" and V_{max} values were calculated from the negative reciprocal slope and the intercept with the abscissa, respectively, of plots of v/S against v (Eadie–Hofstee plots).

Steady-state reduction of cytochrome *c* in the presence of oxidase, TMPD, ascorbate, and O_2 was measured with the Aminco DW2a spectrophotometer in the dual wavelength mode at 550 minus 535 nm. First we recorded the absorbance of oxidase plus cytochrome *c* (cytochrome *c* oxidized), then the increased absorbance on addition of TMPD plus ascorbate (aerobic steady state), and then the further increase (cytochrome *c* reduced) when the aerobic suspension became anaerobic as a result of the O_2 uptake. The experimental conditions were the same as those used in the corresponding O_2 uptake measurements. Any change in absorbance due to endogenous pigments of the oxidase preparation at this wavelength pair as the mixture became anaerobic was assessed by adding 0.75 mM KCN to a similar aerobic suspension. Such changes proved to be immeasurably small in most of the experiments.

Results

Spectrophotometric Method. As previously observed in many laboratories, the oxidation of ferrocytochrome *c* was found to follow a first-order course throughout in different kinds of buffers at pH values between 6 and 7.4. Yonetani & Ray (1965) reported deviations from first-order kinetics with some, but not all, kinds of preparations at pH values above 7.4 (the deviations seem to occur at cytochrome *c* concentrations above about 10 μM). However, with our preparations the reaction also proved to be first order in ferrocytochrome *c* in Tris–cacodylate buffer at pH 7.8, although the rates were relatively low at this pH. Thus, our observations are not in accord with the conclusion expressed by Ferguson-Miller et al. (1979) that first-order kinetics with the spectrophotometric method are seen only under unphysiological conditions. At pH 7.8 the first-order rate constants decreased with an increase in the total concentration of cytochrome *c*, as observed at lower pH values, with sharp decreases up to 0.25 μM cytochrome *c* and then more gradual decreases with higher concentrations

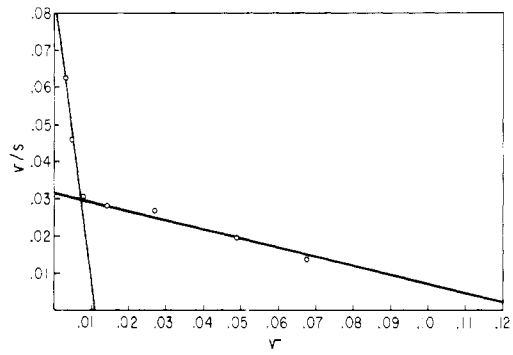

FIGURE 1: Spectrophotometric assays with increasing concentrations of cytochrome *c*. SMP deficient in cytochrome *c* and treated with DOC, containing 0.0026 mg of protein, and 0.0022 μM cytochrome aa_3 were used in each assay in 25 mM Tris–cacodylate buffer, pH 7.8. Data are calculated as v/S against v.

Table I: "Apparent K_M" and V_{max} Values Derived from Polarographic and Spectrophotometric Assays[a]

	SMP treated with DOC		purified cytochrome aa_3	
	polaro.	spectro-phot.	polaro.	spectro-phot.
"high affinity"				
apparent K_M (μM)	0.055	0.133	0.036	0.22
turnover rate[b]	78.2	5.1	17.5	2.3
"low affinity"				
apparent K_M (μM)	1.65	4.17	0.314	3.85
turnover rate[b]	293	59	33.6	13.1

[a] All assays were run in 25 mM Tris–cacodylate buffer (25 mM in cacodylate), pH 7.8. In the polarographic assays TMPD was 0.75 mM and ascorbate was 10 mM. The "apparent K_M" and V_{max} values were derived from plots of v/S against v, as described in Materials and Methods. [b] μM cytochrome *c* s^{-1} at V_{max} divided by the concentration of cytochrome aa_3.

of cytochrome *c*. Plots of v/S against v derived from such data (Figure 1) show two linear parts, with a break at the point obtained with 0.25 μM cytochrome *c*. "Apparent K_M" and V_{max} values calculated from the two lines are found in Table I. With a fixed concentration of cytochrome *c*, the rate constants were linearly related to the concentration of oxidase in the assays.

Polarographic Method. We encountered a number of difficulties in working with the polarographic method.

(1) Cytochrome *c* will adsorb to the electrode membrane, and this can be cumulative. Incubation of the electrode in 0.05 M phosphate buffer with stirring for several minutes before washing it between each assay eliminates this problem.

(2) Care must be exercised to avoid the nonenzymatic O_2 uptake that results from a combination of substances such as heavy metals with ascorbate. Some of our samples of cytochrome *c* and TMPD and all of the samples of sucrose we tested contained substances which gave rise to KCN-insensitive O_2 uptake in the presence of ascorbate. We also found that the use of syringes with metal tips and plungers in introducing ascorbate into the O_2 electrode chamber resulted in erratic effects on the O_2 uptake rates. Such effects have been observed by others.[2] Although the nonenzymatic rates can be measured and subtracted from the final rates, these can make a considerable correction at low rates of O_2 uptake. Since we found that inclusion of 0.25 M sucrose made no difference in the rates obtained in the buffers used, we eliminated it from our assays.

[2] Personal communication from B. L. Trumpower.

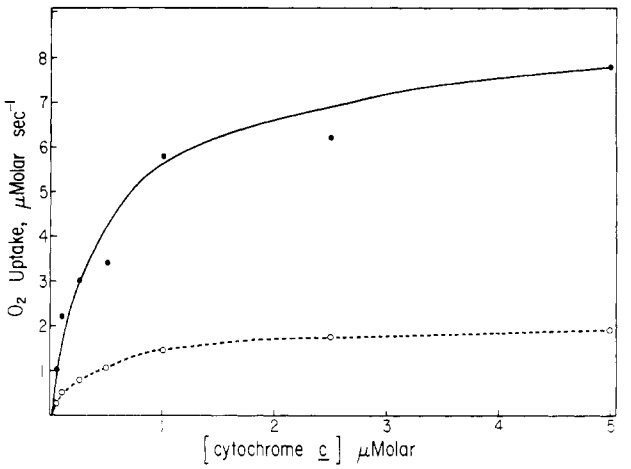

FIGURE 2: Effect of dilution on O_2 uptake by purified oxidase. The purified preparation of cytochrome aa_3 was assayed in 25 mM Tris–cacodylate buffer, pH 7.8: (○) 10 μL of undiluted preparation; (●) 10 μL of 20-fold diluted preparation (multiplied by 20).

(3) We found the greatest difficulty in working with the purified cytochrome aa_3 preparation, where the measured rate was found to be dependent upon the extent of dilution of the preparation with buffer (0.01 M phosphate, pH 7) containing 1% Tween 20 or 80 *before* addition to the reaction mixture (up to 20- to 50-fold dilution). Figure 2 illustrates this effect. Dilution of the preparation before assay, rather than use of a smaller volume of undiluted preparation, increased the activity fourfold. The increase was sometimes greater than this. Dilution of SMP or of SMP treated with detergent had only small effects, if any (<50% increase). With any form of SMP or with diluted or undiluted purified cytochrome aa_3 preparations, a linear relationship was observed between the O_2 uptake rates and the quantity of oxidase in the assays (data not shown).

O_2 uptake rates per cytochrome aa_3 were measured with a number of different kinds of oxidase preparation with increasing concentrations of cytochrome *c* and 0.5 mM TMPD plus 10 mM ascorbate in 25 mM Tris–cacodylate buffer (25 mM in cacodylate), pH 7.8. These conditions gave maximal rates of O_2 uptake; however, in subsequent experiments we employed 0.75 mM TMPD to provide an excess. The rates have been corrected for any O_2 uptake due to remaining endogenous cytochrome *c* or to any nonenzymatic O_2 uptake. With all oxidase preparations tested the rates increased sharply with increasing concentrations of cytochrome *c* up to ~0.1–0.25 μM and then increased more gradually with further increases of cytochrome *c*. When these data were plotted as v/S against v (Figure 3), smooth continuous curves were usually obtained without the sharp breaks observed by Ferguson-Miller et al. (1976) in similar experiments, although the curves with untreated SMP are more nearly like theirs, as is the one of Figure 3. All of the curves show a sharp decrease in v/S at concentrations of cytochrome *c* up to ~0.1–0.25 μM and then less of a decrease at higher levels; this was observed irrespective of the concentration of cytochrome aa_3 in the assays. Even with the curvature of the plots, it is clear that the initial slopes in Figure 3 are similar. Whatever the significance of these slopes, it is the same for the different kinds of oxidase preparations. Somewhat greater variation is evident in the final slopes. "Apparent K_M" and V_{max} values calculated from the initial and final slopes of data obtained in Tris–cacodylate, pH 7.8, with SMP treated with DOC and with a purified preparation are listed in Table I, along with values obtained from the spectrophotometric assays

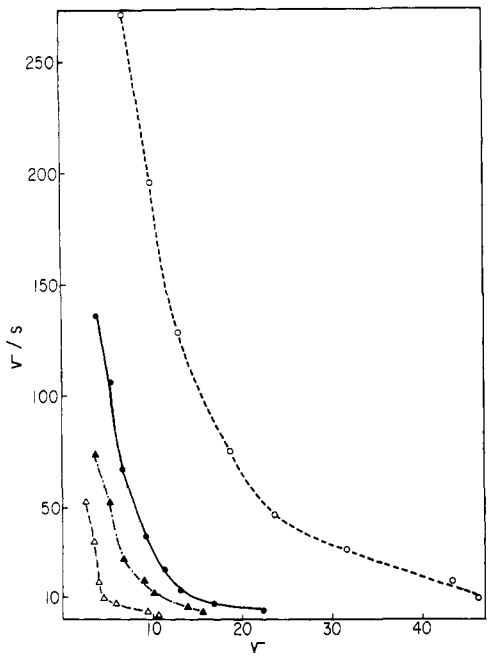

FIGURE 3: O_2 uptake with increasing concentrations of cytochrome *c*. All SMP were deficient in cytochrome *c*. (△) Untreated SMP, 0.056 mg of protein; (○) SMP treated with DOC, 0.028 mg of protein; (●) SMP treated with Triton X-100, 0.056 mg of protein; (▲) purified cytochrome aa_3. All assays were run in 25 mM Tris–cacodylate buffer, pH 7.8. *v* is O_2 uptake s^{-1} divided by the concentration of cytochrome aa_3. Data are plotted as v/S against *v*.

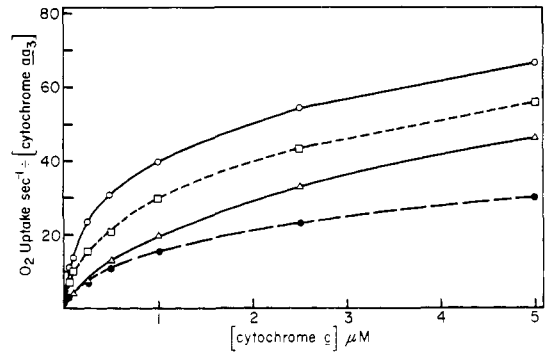

FIGURE 4: Cytochrome *c* deficient SMP treated with DOC assayed polarographically in (○) 25 mM Tris–cacodylate, pH 7.8; (●) 25 mM Tris–cacodylate, pH 6.1; (△) 25 mM phosphate, pH 6.1; and (□) 25 mM borate, pH 7.8. The concentration of cytochrome aa_3 was 0.0226 μM at pH 7.8 and 0.045 μM at pH 6.1.

where similar plots gave two lines. The values derived from the two different methods show considerable differences with any kind of oxidase preparation.

Comparison of Spectrophotometric and Polarographic Assays. Variations of pH or of ionic strength or even of buffer type had quite different effects on the rates measured by the two methods, as illustrated by the data of Figures 4 and 5. O_2 uptake rates with low concentrations of cytochrome *c* were rather low and similar in 25 mM phosphate or Tris–cacodylate, pH 6.1, and were several-fold greater at pH 7.8 in either borate or Tris–cacodylate buffers. However, the spectrophotometrically measured rates with low concentrations of cytochrome *c* were high in phosphate or Tris–cacodylate at pH 6.1 or in borate at pH 7.8, but were low in Tris–cacodylate, pH 7.8. At higher concentrations of cytochrome *c* the spectrophotometrically measured rates in phosphate buffer, pH 6.1, were considerably higher than those in the other buffers. In Figures 4 and 5 the rates are expressed as measured or calculated O_2 uptake rates divided by the concentration of cytochrome aa_3

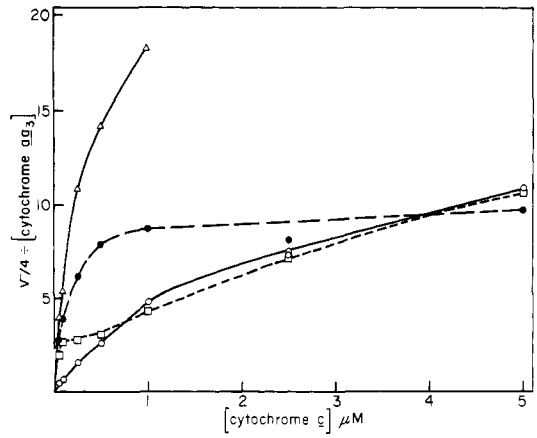

FIGURE 5: Similar SMP preparation and buffers as in Figure 4, but assayed spectrophotometrically. The concentration of cytochrome aa_3 was 0.0018 μM in Tris–cacodylate, pH 7.8; 0.00045–0.0009 μM in phosphate and Tris–cacodylate, pH 6.1; and 0.00045–0.0023 μM in borate, pH 7.8.

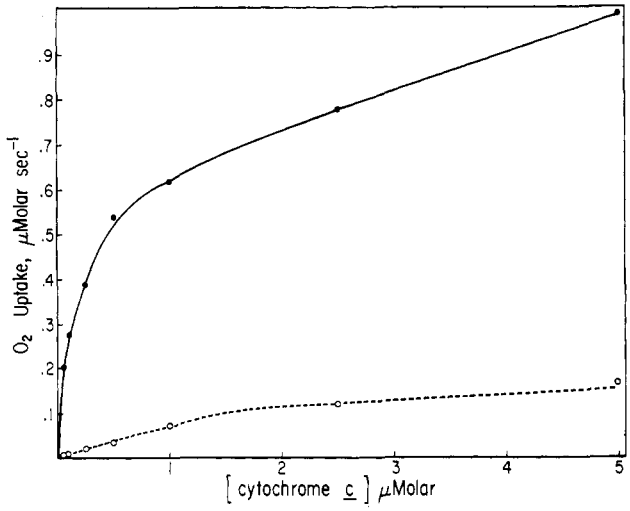

FIGURE 6: Comparison of rates measured polarographically and spectrophotometrically at pH 7.8. (●) Measured O_2 uptake rates in polarographic assays with SMP (cytochrome *c* deficient) treated with DOC, with 0.022 μM cytochrome aa_3. (○) O_2 uptake rates corresponding to spectrophotometrically measured rates calculated for the same concentration of cytochrome aa_3. All assays were made in 25 mM Tris–cacodylate buffer, pH 7.8.

in the assays. In Tris–cacodylate buffers the pH optimum for the spectrophotometric method was around 6 at both high and low concentrations of cytochrome *c*, while that for the polarographic method was 7.8 at low cytochrome *c* concentrations and 7.3 with concentrations as high as 5 μM. The polarographically measured O_2 uptake rates were relatively insensitive to changes of ionic strength; the addition of 0.025 M NaCl to assays in 0.025 M Tris–cacodylate had no or a slightly inhibitory effect, while a similar addition stimulated the spectrophotometrically measured rates (data not shown).

Because of the differential effects of experimental conditions on the two methods, the O_2 uptake rates measured under some conditions were higher than the corresponding rates calculated from the spectrophotometrically measured rates of oxidation of cytochrome *c*. In 25 mM Tris–cacodylate, pH 7.8, at low cytochrome *c* concentrations the rates were as much as 30-fold greater; Figure 6 shows data obtained on simultaneous assay of SMP treated with DOC under these conditions, the only difference being the presence of TMPD plus ascorbate and a fivefold greater level of oxidase (but of the same dilution) in the polarographic assays. At other pH values or in other

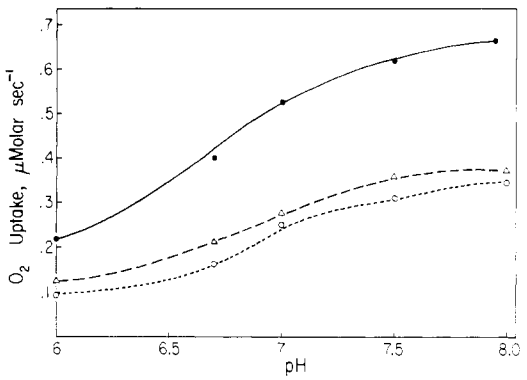

FIGURE 7: O_2 uptake with endogenous and exogenous cytochrome *c*. (○) O_2 uptake with TMPD plus ascorbate and SMP (not deficient in cytochrome *c*) containing 0.114 mg of protein per mL and no added cytochrome *c*. (●) O_2 uptake rates after addition of 0.1 μM cytochrome *c*. (△) Total minus endogenous. Assays were run in 25 mM Tris–cadocylate buffers of varying pH.

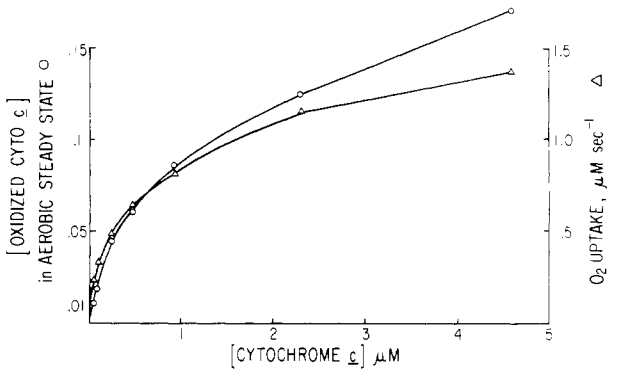

FIGURE 8: (△) O_2 uptake rates measured polarographically with SMP (deficient in cytochrome *c*) treated with DOC containing 0.0226 μM cytochrome aa_3. (○) Concentration of oxidized cytochrome *c* in the aerobic steady state under the conditions of the polarographic assays (0.75 mM TMPD plus 10 mM ascorbate). All measurements were made in 25 mM Tris–cacodylate buffer, pH 7.8.

kinds of buffer, the relationship between the two methods was quite different. For example, in phosphate buffer, pH 6.1, the measured and calculated rates were similar, particularly at low concentrations of cytochrome *c*. Thus, in Tris–cacodylate buffer, pH 7.8, there was considerable extra O_2 uptake measured polarographically, but this was less or absent under other conditions.

Comparison of O_2 Uptake with Endogenous and Exogenous Cytochrome c. O_2 uptake rates of SMP not deficient in cytochrome *c* were measured in the presence of TMPD plus ascorbate with no cytochrome *c* added (endogenous cytochrome *c* only) and compared with the rates which followed on addition of exogenous cytochrome *c*. Figure 7 shows that the rates with endogenous cytochrome *c* and with added 0.1 μM exogenous cytochrome *c* (total minus endogenous) showed similar variations with changing pH in 25 mM Tris–cacodylate buffers. In this experiment the concentration of endogenous cytochrome *c* was 0.043 μM. Similar experiments with several different SMP preparations all showed rates with 0.1 μM exogenous cytochrome *c* to be about equal to the rates with the endogenous cytochrome *c*, which varied between 0.027 and 0.043 μM.

Steady-State Reduction of Cytochrome c in the Presence of TMPD Plus Ascorbate. The extent of reduction of added cytochrome *c* in the presence of TMPD, ascorbate, and O_2 under the conditions of the polarographic assays was measured in the Aminco DW2a spectrophotometer in the dual wavelength mode at 550–535 nm, as described under Materials and Methods. Typical data obtained with a cytochrome *c* deficient SMP treated with DOC are plotted in Figure 8 along with simultaneous measurements of O_2 uptake with identical mixtures. The rates of O_2 uptake follow the same pattern as the concentrations of cytochrome *c* which remain oxidized during the aerobic steady state, particularly at the lower concentrations of cytochrome *c*. Table II shows the concentrations of oxidized cytochrome *c* in the presence of several low concentrations of added cytochrome *c* in a similar experiment. With 0.05 μM added cytochrome *c* the concentration of cytochrome *c* remaining oxidized was just about equal to the concentration of cytochrome aa_3 in the cytochrome *c* deficient SMP treated with DOC used in each assay and then increased to about twice the concentration of cytochrome aa_3 in the presence of 0.25 μM added cytochrome *c*. Similar experiments run in 50 mM phosphate buffer, pH 7, show the same correlation between the O_2 uptake rates and the concentrations of oxidized cytochrome *c* in the aerobic steady state, both of which are low compared to those measured in

Table II: Concentration of Oxidized Cytochrome *c* in Aerobic Steady State in the Presence of TMPD plus Ascorbate[a]

concn of cytochrome *c* added (μM)	concn of oxidized cytochrome *c* in aerobic steady state (μM)
0.05	0.020
0.10	0.032
0.25	0.052
0.50	0.074
1.0	0.092

[a] All assays were run in Tris–cacodylate buffer, 25 mM in cacodylate, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate with SMP (deficient in cytochrome *c*) treated with DOC containing 0.0226 μM cytochrome aa_3 in each assay.

Tris–cacodylate, pH 7.8. However, at pH 6 the concentration of oxidized cytochrome *c* in the aerobic state in the presence of 0.05 μM cytochrome *c* was only about one-fifth that of the concentration of cytochrome aa_3 in the experiments. We were not able to obtain reproducible data with concentrations of added cytochrome *c* below 0.04 μM by using the Aminco DW2a spectrophotometer, and we had difficulty in working at pH 6 with this kind of experiment, apparently due to increased turbidity of the oxidase preparations at this pH.

Discussion

In spite of the extensive use of both spectrophotometric and polarographic methods for assaying cytochrome *c* oxidase, the two methods have not been compared previously under the same experimental conditions with low concentrations of cytochrome *c*. Ferguson-Miller et al. (1978b) investigated both methods, but they claimed that they did not see evidence for a "high affinity" binding site with the spectrophotometric method. However, they worked with cytochrome *c* concentrations between 0.7 and 85 μM and did not report data obtained with the concentrations of cytochrome *c* in the range where the "high affinity" site was evident in the polarographic assays (up to ~0.1 μM). Our comparison of the two methods has revealed some new aspects of the reaction of cytochrome *c* with cytochrome *c* oxidase.

An observation made consistently with both kinds of assay, under all conditions tested and irrespective of the content of cytochrome aa_3 in the assays, was the sharp increase in rate with increasing concentrations of cytochrome *c* up to between 0.1 and 0.25 μM and then the more gradual increase with higher concentrations of cytochrome *c*. Plots of the data in the form v/S against v gave two linear parts in the spec-

trophotometric assays, and in the polarographic assays a break or a bend is present in the plot at the point obtained with 0.1 or 0.25 μM cytochrome *c*. This consistency in the breaks of the plots would argue against the initial and final slopes being measures of the K_D for two binding sites, as suggested by Ferguson-Miller et al. (1976, 1978a), since the relative values should change with varying experimental conditions. It rather appears that the concentration of added cytochrome *c* affects the oxidase activity. The same low concentrations of cytochrome *c* which gave the high relative rates with both assay methods also gave polarographic rates similar to those observed with the endogenous cytochrome *c* of cytochrome *c* sufficient SMP, where diffusion of cytochrome *c* to the oxidase should not be involved.

Ferguson-Miller et al. (1976, 1978a) concluded that the values of "apparent K_M" derived from plots of v/S against v under different experimental conditions are measures of the K_D for binding of cytochrome *c* to the oxidase, although they state that the "slow dissociation of cytochrome *c* from its high-affinity binding to the oxidase is no longer an obligatory step" in the polarographic method but that "dissociation is likely to be rate limiting under many conditions in the spectral assay". The calculations of Errede & Kamen (1978) based on the reaction assayed spectrophotometrically at pH 6 showed that the negative slope of the plot of k' against k'[cytochrome *c*] (the same as v/S against v) was a measure of the K_D of the binding of ferrocytochrome *c* to the oxidase; they also assumed that the K_{eq} for the binding of the ferrocytochrome *c* to the oxidase equalled that for the dissociation of the ferricytochrome *c*–oxidase complex. Since we obtained similar "apparent K_M" values from both the spectrophotometric and the polarographic methods at pH 6, they could be measures of binding constants at this pH. However, different "apparent K_M" values were derived from the two methods in Tris–cacodylate buffer at pH 7.8; thus, under these conditions something different is being measured (see below).

The large excess of O_2 uptake observed under some conditions in the polarographic assays, as compared with rates calculated from the spectrophotometric measurements, can be most simply explained by the formation of a combination of cytochrome *c* and cytochrome oxidase which can be repeatedly reduced by TMPD before it dissociates. Then the extra O_2 uptake is a measure of the turnover of such a complex under the experimental conditions used. This postulate has been expressed by Ferguson-Miller et al. (1978a). However, varying the experimental conditions affects the rates obtained by the two methods in quite different, but not in reciprocal, ways. For example, with low concentrations of cytochrome *c*, rates are high in both in borate buffer, pH 7.8. Thus, the simple assumption that conditions which increase the rate of dissociation of the complex will increase the rate in the spectrophotometric assays and will decrease the O_2 uptake rates does not hold. Ferguson-Miller et al. (1978a) also obtained different effects of pH and buffer type on rates obtained by the two methods, but their data with the spectrophotometric method only apply to reactions at their so-called "low affinity" site, as explained above.

Taken altogether, our data suggest that different combinations of cytochrome *c* and cytochrome oxidase can be formed under different experimental conditions and that under some conditions there is the formation of an especially reactive complex, with either increased interaction between the components or the capacity for more rapid reduction or oxidation of the proper components. The ability to form such a complex varies with the state of the oxidase preparation and the experimental conditions. Optimal rates of O_2 uptake with both endogenous and with added cytochrome *c* were seen at pH 7.8.

Observations of the state of reduction of the added cytochrome *c* during the aerobic steady state under the conditions of the polarographic assays showed a correlation between the cytochrome *c* which remained *oxidized* and the simultaneously measured rates of O_2 uptake. This points to a form of cytochrome *c* which is very rapidly oxidized and which remains oxidized until the O_2 in solution is exhausted and gives further evidence for the turnover of a highly reactive complex between cytochrome *c* and cytochrome oxidase. In the experiment of Table II, 0.05 μM added cytochrome *c* was 40% oxidized during the aerobic state and the concentration of oxidized cytochrome *c* was just about equal to the concentration of cytochrome aa_3 in the reaction mixture. When these measurements were repeated in phosphate buffer, pH 7.0, the O_2 uptake rates also correlated with the concentration of oxidized cytochrome *c* in the aerobic steady state, but the concentration of oxidized cytochrome *c* in the presence of 0.05 μM added cytochrome *c* was only one-fifth that of the concentration of cytochrome aa_3, implying formation of less of the highly reactive complex. TMPD reduced cytochrome *c* less rapidly at pH 7 than at pH 7.8;[3] thus, the decreased oxidation at pH 7.0 cannot be explained by an increased ability of TMPD to reduce cytochrome *c*. The conclusion of Ferguson-Miller et al. (1978b) from studies of the initial kinetics of oxidation of cytochrome *c* at −49 °C that "structural characteristics important for the association of cytochrome *c* and oxidase are also essential for achieving normal rates of electron transfer within the complex" seems to us to be in agreement with our postulate of complexes of different reactivity.

Among the different experimental conditions tested, the turnover rates of the oxidase at V_{max} of the "high affinity" site were highest with the polarographic method in 25 mM Tris–cacodylate, pH 7.8, using SMP treated with DOC, where the oxidase appears to be maximally exposed for reaction with cytochrome *c* (Smith & Camerino, 1963b). Rates up to 100 s^{-1} were obtained with a number of different SMP preparations. This is hardly enough to account for the rates observed with intact mitochondria (Nicholls & Chance, 1974), so perhaps we have not yet found the optimal conditions for the formation of the maximally reactive cytochrome *c*–cytochrome oxidase complex. The maximal rates obtained with the spectrophotometric assays under these conditions are quite low; this appears to be evidence, in agreement with results from other experimental approaches (Erecinska et al., 1975; Davies et al., 1976), that cytochrome *c* does not dissociate from the oxidase during normal electron transport.

The turnover rates at the proposed "low affinity" site with higher concentrations of cytochrome *c* can be as high as 300 s^{-1}. Thus, if there are two sites on the oxidase where cytochrome *c* can bind, electrons can apparently be introduced in tandem, unless it is assumed that the binding of a second molecule affects the turnover of the first.

Different experimental approaches all lead to the conclusion that the binding site on cytochrome *c* for the oxidase is in the area at the top of the heme crevice (Staudenmayer et al., 1976; Smith et al., 1976, 1977; Ferguson-Miller et al., 1978a). It would not be unexpected that multiple attachments would be involved in aligning cytochrome *c* with the oxidase to create the proper arrangement for maximal electron transfer. Thus, changing experimental conditions could have subtle or more profound effects on the ability to form the combination ar-

[3] Unpublished observations.

ranged for maximal electron transfer. Further detailed experiments might reveal the nature of the reactive groups involved.

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Construction and Characterization of Pro α1 Collagen Complementary Deoxyribonucleic Acid Clones†

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ABSTRACT: Double-stranded cDNA to embryonic chick procollagen mRNAs was synthesized by using the avian myeloblastosis viral reverse transcriptase. After ligation to chemically synthesized decanucleotides containing the *Hin*dIII restriction site, these double-stranded cDNA sequences were inserted into the *Hin*dIII site of pBR322. The recombinant plasmids were then used to transform *Escherichia coli* χ1776 and recombinants containing procollagen cDNA sequences identified by colony hybridization to ^{32}P-labeled procollagen cDNA. In addition to the three pro α2 collagen cDNA clones described recently (Lehrach et al., 1978) three additional recombinant plasmids pCg26, pCgl, and pCg54 with inserts 640, 850, and 1100 base pairs long have been identified. Their sequence homology has been determined by restriction mapping and by DNA sequencing. pCg54 has been positively identified as a pro αl collagen cDNA clone by partial DNA sequencing of its ends: it has a sequence coding for residues 811–858 in the chick α1 chain near one end. pCg54 overlaps pCgl by 250 nucleotides and together these extend about 1500 nucleotides from the poly(A) end of pro α1 collagen messenger RNA.

Collagen is the most abundant protein in vertebrates, constituting 30% of the protein in mammals. The primary and secondary structure of collagen, its biosynthesis, and its assembly into fibrils have been well characterized (Fessler & Fessler, 1978; Miller, 1976). Little is known, however, of the mechanisms regulating the expression of the five or more collagen genes despite the fact that the onset of collagen synthesis is an essential step in differentiation and aberrations in collagen gene expression are the cause of some human diseases such as Osteogenesis Imperfecta and Ehlers Danlos

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