The Decline of Molecular Activity of Cytochrome Oxidase during Purification*

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SUMMARY

The losses of molecular activity which take place when cytochrome oxidase is purified from bovine heart muscle particles by the procedures of Yonetani ((1960) J. Biol. Chem. 235, 845) and of Fowler et al. ((1962) Biochim. Biophys. Acta 64, 170) have been examined. Bovine heart muscle particles (about 1 n mole of heme a per mg of protein) and oxidase-rich particles (about 4 n moles of heme a per mg of protein) treated with deoxycholate under optimal conditions, had molecular activities (MAo, max) at (at infinity high concentration of cytochrome c, as electrophotometrical determined in 0.05 M phosphate buffer, pH 7.0, 25°) in the range of 530 to 580 s⁻¹.

MAo, max, determined under identical reaction conditions, declined with successive steps in the Yonetani and Fowler et al. purification procedures for cytochrome oxidase, to considerably lower values for the finally isolated enzymes, while Kass° stayed relatively unchanged. Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate, showed no evidence of essential component removal during purification, that might account for decreased MAo, max values. The accumulation of an endogenous inhibitor in the course of preparation was also shown to be an unlikely cause for the observed decline of MAo, max.

Treatment with limited quantities of dodecyl sulfate led to stimulation of activity of Yonetani's preparation. Similar treatment of Fowler's preparation and of deoxycholate-treated, oxidase-rich particles resulted only in decreased molecular activity. Dodecyl sulfate appeared to be an uncompetitive inhibitor of cytochrome oxidase.

The aerobic ferrocytochrome c oxidation catalyzed by deoxycholate-solubilized particulate oxidase exhibited apparent first order kinetics over at least 99% of the reaction course. Purified oxidase preparations showed deviations from first order (concave semilogarithmic reaction plots) at low remaining substrate concentrations. This could not be ascribed either to spontaneous activation in the reaction mixture or to unequal affinities of the oxidase for ferrous and ferric forms of cytochrome c. Reversible unproductive binding of ferrocytochrome c with a fraction of the oxidase in the purified preparations explains this. Deoxycholate-treated oxidase particles exhibited a largely oxidized 605 nm band during the aerobic steady state in the system containing ascorbate, cytochrome c, oxidase, and O₂, whereas Yonetani's enzyme appeared appreciably reduced. This indicates a blockade of the electron transfer from a to a₃ in the inactive or latent portion of the oxidase preparation.

It appears from these observations that some portion of the original membrane-bound cytochrome oxidase may be transformed during purification into catalytically inactive or latent species, possibly by reorganization of the original multimeric phospholipid-protein complex in a portion of the purified enyzmes. Various physical, chemical, and catalytic complexities which have been observed with chemically purified cytochrome oxidases may have their origins in molecular heterogeneities.

Cytochrome oxidase (EC 1.9.3.1) participates in the respiratory chain of the inner mitochondrial membrane as electron transfer catalyst between ferrocytochrome c and molecular oxygen. This membranous enzyme plays a role in energy conservation and related phenomena such as respiratory control and ion translocation (e.g., Refs. 1 and 2). Investigations of the oxidase in its pure state are of fundamental interest because they offer a greater promise for complete resolution of the structure-function relationship with presently available techniques than the complementary approach of studying the intact membrane-bound electron transfer system. However, such work can be meaningful only if the essential structural and functional characteristics of the oxidase are not altered by isolation.

A number of observations suggest that structure and function is maintained during extraction and purification: (a) similar reaction order and dependence of the rate constant on the sum of the concentrations of ferrous and ferric cytochrome c of exogenous-cytochrome c oxidation catalyzed by oxidase in situ (3-6) and of enzyme detached from membrane (7) and further purified (6, 8-12); (b) sensitivity of purified cytochrome oxidase to respiratory inhibitors such as cyanide (e.g., Ref. 13), azide (e.g., Ref. 14) and carbon monoxide (15); (c) similar effects produced by changes
in pH (7, 16), ionic strength (7, 17), or temperature (4, 7) on soluble cytochrome c oxidation catalyzed by both particulate and soluble cytochrome oxidase; (d) similarity of the visible absorption spectra in both states with respect to peak positions (compare 18 with, e.g. Ref. 19) and intensity, the latter at least for the reduced-CO minus reduced difference spectrum (20); (e) exact correspondence between the photochemical action spectra and the reduced-CO minus reduced difference spectrum (20); (f) almost identical CO-binding capacity of the enzyme on Keilin-Hartree particles and in the purified state (20); (g) identical nature of reactant and product in both solubilized and membrane-bound catalytic systems; and, (h) reconstitution of Site III of oxidative phosphorylation with purified cytochrome oxidase (22-24).

Quantitative comparisons between the specific activities in terms of heme a (as measured by spectral parameters) of membrane-localized and purified cytochrome oxidase at first tended to support the functional intactness of purified systems (8, 25-27). However, subsequent work by Smith and Camerino (28) and by Smith and Newton (7) has shown that membrane-bound cytochrome oxidase is incompletely exposed for reaction with soluble cytochrome c, because the rate of O2 consumption can be raised, either by passing electrons through the endogenous cytochrome c, or by detergent treatment under specific conditions. Thus, purified preparations previously claimed to display adequate activity are in fact considerably less than maximally active (28). Furthermore, an exhaustive survey of specific activities (29) reveals large variations among different preparations.

This study was initiated after the unexpected observation was made that two distinct preparative procedures (30, 31) yielded enzymes exhibiting magnetic interactions of opposite sign, indicating an effect of preparative procedure on the isolated structures. In addition, we wished to test the assumption that purification of cytochrome oxidase results in an enzyme with homogeneous chemical and functional properties (32). We report our observations pertaining to the molecular activity of cytochrome oxidase of disrupted mitochondrial particles and of stages obtained in the course of two frequently used isolation procedures (19, 30). Finally, we discuss the nature of the changes which result in the loss of molecular activity in purified oxidase preparations.

**EXPERIMENTAL PROCEDURES**

**Preparative Procedures**—The starting material for cytochrome oxidase preparations was beef heart muscle mitochondrial particles (20). Particles for the Yonetani preparation were washed once with 1 liter of H2O, resedimented by centrifugation as before, and then suspended in 0.02 M phosphate buffer, pH 7.4, with a glass-Teflon homogenizer. Heart muscle particles to be subjected to the purification method of Fowler et al. (30) were dispersed in 0.1 M phosphate buffer, pH 7.4, containing 0.25 M sucrose, and spun in a Beckman centrifuge rotor 21 at 21000 rpm for 2 hours, then resuspended by homogenization in the Tris-sucrose-histidine medium used in this procedure. From 1.0 to 1.4 nmoles of heme a were present per mg of protein, and the oxidase activity was very high after deoxycholate treatment according to Smith and Camerino (28) (see below).

Preparative procedures were prepared from the particles by Yonetani's procedure (19) except for minor modifications: (a) phosphate buffer containing 1% sodium cholate was used instead of 2% (33) to avoid NH4 cholate precipitation; (b) we progressively narrowed the (NH4)2SO4 fractionation ranges to 35 to 50%, 25 to 35%, 27 to 35%, and, finally, 27 to 34% (cf. Ref. 9); thus, four instead of six fractionations sufficed to produce spectrally pure oxidase; (c) the final preparation dissolved in 0.1 M phosphate buffer, pH 7.4, containing 1% Tween 80 (replacing Emasol 4130 used by Yonetani) was dialyzed for 10 hours against 10 times diluted solvent to remove cholate (34).

The purification procedure of Fowler et al. (30) was applied to heart muscle particles instead of mitochondria (6, 20). A slightly different range of fractionation (i.e. 0.17 ml of saturated (NH4)2SO4 solution per ml of dialysate followed by adding 0.04 ml per ml of dialysate) was adopted to be in agreement for a high yield of particulate-rich preparations were prepared from heart muscle particles by the first deoxycholate-KCl treatment of the Fowler procedure (30) which extracts the major part of cytochromes b and c1 and a substantial portion of the protein, but leaves the oxidase attached to particulate matter (the red-green split (35, 36)). Green-brown particles thus prepared were washed with and resuspended in Tris-sucrose-histidine buffer. Their concentration of oxidase was adjusted to 10 mg of protein per ml. The heme a content was in the range of 3.8 to 4.0 nmol of protein. Preparations of particulate or purified cytochrome oxidase, and samples taken at different stages in the course of purification were stored in sealed containers under N2 atmosphere in a liquid N2 dewar. It was shown that the operation of freezing (quickly in liquid N2) and thawing did not materially affect the molecular activity of the oxidase in these samples. We are not aware of any contact of our enzyme preparations with tobacco smoke.

**Materials**—Cytochrome c (type V from bovine heart, 98% pure, Lot 1193-7190) was purchased from the Sigma Chemical Company. This sample of cytochrome c was shown to be free of inhibitory substances (37) by the exact coincidence of rate measurements (at 240 mW power) of ferrocytochrome c concentration of oxidase (treated, oxidase-rich particles) before and after dialysis of the cytochrome c against distilled water for 1 week. Reduced cytochrome c (95 to 98%) was prepared by dithionite addition and anacrobic gel filtration (10). It was kept frozen at liquid N2 temperature in sealed vials under N2 atmosphere prior to use.

Oxidized cytochrome c (99%) was obtained by ferrocyanide treatment followed by gel filtration in a similar manner. It was stored at 0° before use. A sample of oxidized cytochrome c (92.9 μM final) in 0.05 M phosphate buffer, pH 7.0, showed a biphasic course of reduction by 6.7 μM ascorbate with 1.02 μM participating in the slow phase. The latter amount to only slightly more than the fraction of the total cytochrome (i.e. 0.059 or 0.95 μM) which may be expected to reduce slowly at pH 7.0 on the basis of the pH dependent slow equilibrium of two conformers of cytochrome c, an acidic reducible form and a basic irreducible form, with a pK of 9.0 (38). Thus our cytochrome c was essentially uncontaminated by oligomeric and/or other non-native species for which this reduction by ascorbate proceeds slowly (39).

Oxidized phosphate was prepared with primary and secondary sodium salts. The sources of these and other chemicals, except those mentioned below, were described in a previous paper from this laboratory (40). Ammonium sulfate, proanalysis grade, was obtained from Merck and Company; Tris-HCl from Sigma Chemical Company; and SDS (recrystallized from ethanol) from Matheson Coleman and Bell and from Serva Fein Biokemikalien.

**Methods**—Absorption spectra, spectrophotometric oxidase assays, and observations on the redox state of the cytochromes were performed with a Cary model 14 recording spectrophotometer equipped with a thermostatted cell holder and, when necessary, with a sensitive 0.1-0.2 mA slide wire. The concentration of heme a in cytochrome oxidase was measured from the reduced minus oxidized dichroic difference spectrum using 1 cm path length cuvettes of 4 cm3 (44) for the wave length pair 605 to 650 nm. For heart muscle particles and for intermediate fractions which still contained cytochromes b and c1 a procedure was used that corrected for the interference of these cytochromes at the wavelength of peak absorbance aa (41).

Cytochrome b concentration was calculated from the (dithionite) reduced plus CO versus reduced difference spectra of the oxidase using Δε = 210 mM cm⁻¹ at the wave length peak 425.5-445 nm (20). Sufficient time was allowed for these spectra to develop to their maximal deflection. The concentration of cytochrome c was determined from the absorbance difference at 550 nm between (dithionite) reduced and (ferricyanide) oxidized solutions, Δε = 21.0 mM cm⁻¹ (42).

1 W. H. Vanneste and A. Ehrenberg, to be published.
Protein was estimated by the biuret method (43) as modified by Yonetani (9) and Smith and Camerino (28). Bile salts were determined by the method of Mosbach et al. (44).

The transitions to and from the aerobic steady state in the system,

\[
\text{ascorbate} \rightarrow \text{cytochrome } c \rightarrow \text{oxidase} \rightarrow \text{O}_2,
\]

were followed spectrophotometrically at 550 or 521 nm (cytochrome c) and at 605 nm (cytochrome oxidase). An open cell with a 1-cm light path was used in experiments designed to detect activation of Yonetani's enzyme in the reaction mixture by following the approach of cytochrome c oxidoreduction at 550 nm to its steady state value after introducing oxidase into the system. Reaction conditions were the same as for the spectrophotometric activity assay (see below) except for the presence of ascorbate. Transition from the aerobic steady state to the anaerobic state on the other hand was observed in a stopped 10 cm cylindrical cell as needed for sensitive measurements of the redox state of low concentrations of cytochrome oxidase at 605 nm. The reaction mixtures were prepared in an Erlenmeyer flask prior to transfer to the observation cell. In order to be able to account for interference of 606 nm due to cytochrome c, which changes from partially reduced to fully reduced when O2 is depleted, we determined ΔA at this wavelength in the difference spectrum (reduced minus oxidized) of cytochrome c, 1.20 mm−1 cm−1. The increase of the reduced cytochrome c concentration concomitant with the transition to anaerobiosis was obtained from separate observations, under identical reaction conditions, of the change in A at 521 nm using ΔA = 0.26 mm−1 cm−1.

Activity of cytochrome oxidase was measured spectrophotometrically by observing the course of oxidation of ferrocytochrome c at 550 nm in air-saturated 0.05 M phosphate buffer, pH 7.0. The sample of oxidase added was contained in 0.050 ml unless mentioned otherwise. The final volume was 3.00 ml and reactions were run at 20 ± 0.1°C. The apparent first order rate constant, kf, was determined, either from the slopes of the linear portion of the semilogarithmic plots of A1 - A5 against time, or from rate measurements directly on the spectrophotometric reaction curves near zero time. The A5 reading was taken after at least 10 reaction half-times had elapsed or after a few small grains of K3Fe(CN)6 had been stirred into the reaction mixture. The amount of oxidizing agent used did not contribute noticeably to the A5 absorbance. Except for the inhibition experiments with ferricytochrome c (see legend of Fig. 9), the initial velocity, vo, was calculated by multiplying k by the total concentration of cytochrome c, thus disregarding the fact that a very small percentage of cytochrome c was already in the oxidized state before the reaction was started. The molecular activity at zero time, MAo, was obtained by dividing vo by the concentration of cytochrome c present. The cytochrome oxidase concentrations in order to obtain values for MAo,max and Ks,app.

The rate equation for cytochrome oxidase (Equation 1) satisfies several enzymic mechanisms (4, 10, 12, 46, 47). In the absence of evidence in favor of one to the exclusion of the others, interpretation of MAo,max and Ks,app in terms of rate or equilibrium constants for discrete steps has only a limited significance.

**RESULTS AND DISCUSSION**

**Characteristics of Our Oxidase Preparations**

The visible spectra, spectroscopic purity characteristics, and heme a to protein ratios of our purified preparations were similar to those reported by other investigators (9, 14, 30, 31) and they may therefore be considered to have very nearly identical compositions. The molar ratios of cytochrome a3 to heme a were typically 0.50 and 0.48 for heart muscle particles and oxidase-rich particles, respectively, and 0.47 and 0.50 for Yonetani and Fowler preparations.

**Molecular Activities of Heart Muscle Particles and Oxidase-rich Particles after Treatment with Deoxycholate**

The cytochrome oxidase activity of our heart muscle particles was stimulated by deoxycholate (Fig. 1), confirming Smith and Camerino (28). Optimal activation required about 1 mg of deoxycholate per mg of protein, the inset of Fig. 1 indicates the necessity for adding deoxycholate in this ratio to sufficiently concentrated suspensions of particles. Cytochrome c, 53.1 μm was employed in the activity test. Since this concentration is nearly saturating for the particles treated with deoxycholate (Ks,app under optimal conditions = 5.2 μm), but incompletely so for untreated particles (Ks,app = 30 to 40 μm (6)), the rise in MAo at infinitely high concentration of cytochrome c with increasing ratios of deoxycholate to protein should be slightly less marked. MAo,max for this batch of optimally disrupted particles was 550 s−1 when assayed at 4.0 nm cytochrome a. Under comparable conditions of solubilization and activity determination, Smith and co-workers (7, 28, 37) found values for the "maximal turnover number," around 350 s−1. The discrepancy with our
The conditions explored for oxidase-rich particles have been incorporated in a standard treatment for exposing the enzyme optimally to reaction with soluble cytochrome c. Two volumes of ice-cold 5% deoxycholate (of neutral pH) and 2 volumes of saturated KCl at room temperature are added to 5 volumes of ice-cold particle suspension and left at 0°C for 2 min before diluting as required with ice-cold 1% Tween 80-phosphate buffer, 0.1 mM, pH 7.4. The activation takes place instantaneously. Prolonged treatment with deoxycholate plus KCl under the standard conditions results in a loss of MA during the first 3 hours. About 70% of the original MA remains for at least 16 hours. MA,max but not Kapp is affected in this process. Immediate dilution of the mixture with Tween 80-phosphate buffer delays the onset of activity loss for at least 3 hours. Values of MA,max and Kapp obtained with treated oxidase-rich particles are shown in Table I. As expected, the molecular activity of treated oxidase particles is dependent on the ionic strength and the pH of the medium. At pH 6.8 and an optimum μ of 0.111, an MA,max of 830 s⁻¹ and a Kapp = 13.0 μM was observed.

The heart muscle mitochondrial particles treated with deoxycholate did not give a noticeably different MA when diluted with Tween 80-phosphate buffer instead of water except when assayed at low enzyme concentrations (cf. Fig. 4). In the case of pre-treated oxidase-rich particles, adding Tween 80 to the mixture for activity determination did not further increase MA.

Smith and Camerino (28) state that their value (350 s⁻¹) for the molecular activity at infinite cytochrome c of Keilin-Hartree heart muscle particles pretreated with deoxycholate represents the maximal obtainable turnover rate because it agrees with the rate of cytochrome c oxidation by 16 μM O₂ at 25°C in ascites tumor cells (48). However, since much higher levels of O₂ are employed in the assay procedure with soluble cytochrome c, and since the reaction rates are independent of O₂ down to quite low concentrations (cf. zero order polarographic O₂ traces, e.g. Ref. 28), the rate in the system under consideration here is probably limited by some reaction other than that between a₃ and O₂.

Recently, several mechanistic models for the terminal segment of the respiratory chain have been proposed to fit the kinetics of the reduced membrane-bound components responding to an oxygen pulse (49). A number of satisfactory models exhibit rate constants for intercomponent electron transfer compatible with the high molecular activity of cytochrome oxidase which we observe. Such is the case with the scheme that includes both forward and reverse oxidation-reduction steps but an essentially irreversible oxygen reaction. It seems premature to decide whether or not the MAₘₘₗₗₜₜ of solubilized particles corresponds to the maximally attainable molecular activity since evidence is lacking as to the complete molecular dispersal and homogeneity of the oxidase. That is, we have no proof that εₐ₉/εₐ₉ = 1. Accordingly, the highest molecular activity level obtained by deoxycholate treatment is denoted "optimal-deoxycholate-MA" to imply possibly restrictive conditions under which it is observed.

**Molecular Activities of Cytochrome Oxidase in Initial, Intermediate, and Final Fractions of Preparations**

Results obtained with successive cytochrome oxidase fractions of the Yonetani preparation have been plotted according to

![Graph](image-url)
FIG. 3. Effect of purification on the kinetic parameters of cytochrome oxidase (Yonetani preparation). The method and conditions for molecular activity measurements are described under "Methods." ▽, supernatant at 35% (NH₄)₂SO₄ saturation in 1st fractionation, [aₐ] = 1.85 nM; □, precipitate of second fractionation redissolved, [aₐ] = 1.82 nM; △, precipitate of third fractionation redissolved, [aₐ] = 1.73 nM; ○, final Yonetani preparation after dialysis, [aₐ] = 12.5 nM; ♦, deoxycholate-treated heart muscle particles, [aₐ] = 1.67 nM. Cyt. c, cytochrome c.

Equation 6 and fitted with straight lines by means of the least-squares method (Fig. 3). Kₐₐₚₚ and Mₐₐₕₕ max values are tabulated in Table I. The fractions exhibit a reasonably constant Kₐₐₚₚ, yet a strongly and progressively declining Mₐₐₕₕ max. Molecular activity data (at nearly saturating cytochrome c concentrations) are depicted in Fig. 4 as a function of the enzyme concentration in the assay, for two fractions and the final product of the Fowler type preparation.

Comparison with the optimal-deoxycholate-MA of heart muscle particles in Fig. 3 and Table I shows that even the first fraction in the Yonetani preparation has a reduced molecular activity. Since the ratio of bile salt to protein (1 to 1.5 mg per mg) and the protein concentration (about 15 mg per ml) in the step leading to this fraction are adequate for optimally increasing the accessibility of the oxidase, the reason must be sought in the salt effect of Smith and Newton (7), as 35% saturation of (NH₄)₂SO₄ is employed. On the other hand, effects that cause the further decline of MA in later steps may operate during the 2-hour incubation period before obtaining this fraction.

In contrast, the first solubilized fraction of the Fowler preparation is very nearly optimally active. The slight discrepancy with the treated heart muscle particles apparent in the MA diagram (Fig. 4) is accounted for by the unequal Kₐₐₚₚ values (Table I). However, further steps, in particular the dialysis, again cause a decline of MA.

The MA values for our final preparations are consistent with published values. Yonetani determined maximal turnover rates on the basis of heme a of 30 s⁻¹ (50), 50 to 60 s⁻¹ (9), and 60 s⁻¹ (10) under conditions of pH, p, and T fully comparable with ours. Fowler and coworkers (30) found Vₐₐₕₕₕₕ max = 136 moles c min⁻¹ mg⁻¹ which recalculated to our units gives Mₐₐₕₕ max = 252 s⁻¹. Considering that their value was estimated at pH 6.0, we may conclude that our data are in agreement. Van Buuren et al. (6) reported a Mₐₐₕₕ max of 240 s⁻¹ determined polarographically at pH 7.4 for a modified Fowler preparation. The much larger values in the range from 400 to 750 s⁻¹ measured under similar conditions by Mason and Ganapathy (40) may have been due mainly to a shortened time of dialysis in their modification of the Fowler method.

A slight but consistent rise in molecular activity takes place with dilution of the oxidase in the assay. This may be related to the dilution with Tween 80-phosphate buffer before measure-

### Table I

Kinetic parameters of oxidase preparations and of successive fractions in Yonetani method of purification

<table>
<thead>
<tr>
<th>Preparation or fraction</th>
<th>[Cytochrome aₐ] in the activity determination</th>
<th>Kₐₐₚₚ</th>
<th>Mₐₐₕₕ max</th>
<th>aₚₚₚ</th>
<th>pμM</th>
<th>μM</th>
<th>s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycholate-treated heart muscle particles (preparation 701222)</td>
<td>1.67</td>
<td>5.2</td>
<td>542</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxycholate-treated oxidase-rich particles (preparation 710606)</td>
<td>2.22</td>
<td>13.5</td>
<td>576</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yonetani preparation (preparation 710318)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Supernatant at 35% saturation in 1st (NH₄)₂SO₄ fractionation</td>
<td>1.85</td>
<td>6.7</td>
<td>341</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate of second (NH₄)₂SO₄ fractionation redissolved</td>
<td>1.82</td>
<td>4.3</td>
<td>185</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate of third (NH₄)₂SO₄ fractionation redissolved</td>
<td>1.73</td>
<td>6.6</td>
<td>110</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate of fourth (NH₄)₂SO₄ fractionation redissolved and dialyzed</td>
<td>12.5</td>
<td>5.5</td>
<td>74</td>
<td>2.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preparation of Fowler et al. (preparation 700701)</td>
<td>1.6</td>
<td>5.0</td>
<td>203</td>
<td>1.3</td>
<td></td>
<td></td>
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</tr>
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</table>
ment since, as appears from Fig. 4, deoxycholate-treated heart muscle particles diluted with water failed to show this characteristic. We will return to this phenomenon in a subsequent paper. At any rate the effect of dilution seems to indicate that we are not yet observing the maximal molecular activity for the oxidase.

Nature of Change Leading to Loss of Molecular Activity during Purification

Consideration of Equations 4 and 5 shows that a decline of molecular activity during purification accompanied by small changes, if any (Yonetani preparation), of $K_0^{49}$ would most simply arise from a decreasing $e_{a4}/[a_3]$ ratio. In the next sections we will attempt to find proof for a loss of $e_{a4}$ relative to $a_3$ in the isolated oxidase and try to evaluate several possible reasons for it.

Removal of Essential Protein Constituent—A comparison in quantitative terms of electrophoretic polypeptide patterns in an SDS-containing medium of the preparations of Fowler and Yonetani with that of oxidase-rich particles showed no evidence of component extraction correlating with the observed differences in $MA_0$, max. It appears impossible at present to identify with certainty any of the six major components with functional (heme or copper polypeptides) or structural constituents of the oxidase. Some of the components may be merely adventitious, so that one cannot deduce an unambiguous subunit structure from the data. The recent claim by Komai and Capaldi (51) that cytochrome oxidase is built up with 11,500 and 14,000 dalton polypeptides is qualified by the extremely low activity of their preparation. The specific activity quoted by these authors can be recalculated as an $MA_0$ value of approximately 18 s$^{-1}$ (at 38°, pH 7.0, 0.1 M phosphate, 22 μM cytochrome c). Under similar reaction conditions deoxycholate-treated particles manifest an $MA_0$ around 700 s$^{-1}$. The active oxidase in their “highly purified” sample may be associated with less than 3% of the total heme. This low amount of enzymic protein in the functional state would be unnoticed in the electrophoretic pattern.

Accumulation of Inhibitor during Preparation—It seems improbable for several reasons that the low activities of purified oxidase are due simply to inhibition by bile salts as is frequently suggested. (a) The concentrations of cholate present during the activity measurements of fractions in the Yonetani preparation method (Table I) varies between $9 \times 10^{-4}$ and $1.5 \times 10^{-3}$ μM whereas about $2 \times 10^{-4}$% is needed to inhibit purified oxidase by 50% (8, 52, 53). (b) The final enzyme preparation of Fowler et al. contains less deoxycholate, yet displays only about half as much maximal $MA_0$ as treated oxidase-rich particles. (c) Addition to solubilized oxidase particles of a sample of Fowler's preparation denatured at pH 12.4 (a treatment that was shown to be without effect on the bile salt) in molar ratios of 20 and 80 to 1 on a heme basis, does not measurably diminish the MA of the particles. (d) As illustrated in Table II, the activities of Yonetani's enzyme preparation and deoxycholate-treated oxidase-rich particles are additive. It is immaterial whether Yonetani's oxidase is mixed with the particles during the deoxycholate treatment or after the dilution with Tween 80-phosphate buffer.

The latter result excludes not only bile salts, but any compound accumulating in the course of Yonetani's purification procedure that would act as an inhibitor by virtue of an equilibrium combination with cytochrome oxidase, on condition that this equilibrium becomes established reasonably fast in the forward and reverse directions (i.e. taking less than 1 min) and is not greatly shifted towards complexation with less than 1 μM concentration of free inhibitor. Van Buuren et al. (6) have considered a modification of mechanism IV of Minnaert (4) allowing inactive complexes to form between an endogenous inhibitor (concentration $a_3$) and $E$, $E_a$, and $E_P$. This leads to a dependence of $1/MA_0$, max on $1/ae/K_i$ where $K_i$ is assumed to be equal for the three forms of the enzyme. If this type of inhibition were causing the decreased MA of Yonetani's preparation then a comparison of $MA_0$, max for this preparation with the optimal-deoxycholate-MA would suggest a ratio of $ae/K_i$ around 5. The evidence presented in Table II then shows that the endogenous inhibitor should have a stoichiometric ratio of about 1 (since when a $\geq 1$,$e/K_i \leq 5$ and the fraction combined $\geq 0.8$), otherwise it would have affected the activity of oxidase-rich particles. A special kind of endogenous inhibition that might either satisfy the above requirements or be essentially irreversible under the assay conditions is aggregation into inactive polymers, a possibility which will be the subject of the next section.

The findings in Table II also make it unlikely that a transfer of inhibitory substances from the purified oxidase of Yonetani to cytochrome c causes the low MA (37).

Removal of Phospholipid and/or Aggregation—Cytochrome oxidase prepared by the method of Yonetani has a low lipid content (3). Morrison et al. (54) estimated only 1% phospholipid in the preparation of Horie and Morrison (55) for which the method of purification is quite similar to that of Yonetani (18). On the other hand, the isolated enzyme of Fowler et al. (30) contains a full complement, i.e., about 30%, of phospholipid (56, 57). Irrespective of their lipid content, both preparations exhibit molecular activities that are much lower than optimal.

The observation by several authors (56-59) that the removal of phospholipids results in a low activity with soluble cytochrome $c$ and that reconstitution under proper conditions leads to a substantial or total regain of the original activity of the preparations appears consistent with a requirement for phospholipids in the catalytic function of cytochrome oxidase but does not reveal how they are involved. Recently, studies employing high resolution electron microscopy have substantially clarified the role phospholipids play in controlling the aggregation state of isolated oxidase in conjunction with detergents, ionic strength, pH, and the state and concentration of the preparation (59-65). Since conditions leading to aggregation or membrane formation of cytochrome oxidase result in a low specific activity with exogenous cytochrome $c$ (50, 66, 67) ascribable to restricted accessibility for substrate and/or structural effects on the enzyme itself, it may

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**Table II**

<table>
<thead>
<tr>
<th>Type of oxidase</th>
<th>$\mu M/s$</th>
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<tbody>
<tr>
<td>Yonetani's preparation (1)</td>
<td>0.343</td>
</tr>
<tr>
<td>Deoxycholate-treated oxidase-rich particles (2)</td>
<td>1.76</td>
</tr>
<tr>
<td>(1) + (2); Yonetani's enzyme added during the deoxycholate treatment</td>
<td>2.31</td>
</tr>
<tr>
<td>(1) + (2); Yonetani's enzyme added after the dilution</td>
<td>2.17</td>
</tr>
</tbody>
</table>

$[\text{cytochrome c}] = 26.7 \mu M; [\text{cytochrome a}_3] = 4.20 \mu M$, respectively, for the Yonetani preparation and the treated particles. Other conditions are described under “Methods.”
Methods, except for the addition of NaCl instead of KCl to largely reflect their influence on the physical state of isolated beads. It was hypothesized that the effect of phospholipids on the activity avoidance of potassium dodecyl sulfate. Final concentration of cytochrome c in the assay: 1.3 nM. Cyt c, cytochrome c.

be hypothesized that the effect of phospholipids on the activity largely reflects their influence on the physical state of isolated oxidase. A notable exception to this would seem to be the tightly bound cardiolipin fraction (57).

Both the Yonetani and Fowler type of preparations are apt to aggregate as evidenced by electron microscopy (61, 63). Unfortunately, information as to the aggregation state of the oxidase under the conditions proper to the assay with soluble cytochrome c, i.e., freshly diluted oxidase at mM concentrations, is not obtainable with presently available techniques. Hence, an indirect approach was made consisting of attempts to raise the activity of the oxidase preparations by treatment with graded amounts of a dispersing agent, SDS, under conditions that cause a de-polymerization of the oxidase preparation of Okunuki to a 16.6 S compound (68). SDS was found to effect an increase of the molecular activity of purified oxidase. Investigations are in progress that aim at finding a detergent that would imitate SDS only in its stimulatory role. Additional studies are required also in agreement with Orii's and Okunuki's observations with the Okunuki enzyme (67, 68). In contrast, a gradual decline of MA with increasing SDS concentration was observed for Fowler's preparation and the same effect was manifested by deoxycholate-treated oxidase-rich particles. It was shown that SDS behaves as an inhibitor of the uncompetitive type toward cytochrome oxidase activity (Fig. 5), which may be readily interpreted in terms of Mechanism IV of Minnaert (4) by proposing the combination of SDS with enzyme-substrate and enzyme-product complexes. Under the conditions of our activity determinations the inhibition constant has a value of \(1.3 \times 10^{-6}\) M. The SDS effect thus clearly involves overlapping effects of stimulation and inhibition which obscure its true potential for restoring the molecular activity of purified oxidase. Investigations are in progress that aim at finding a detergent that would imitate SDS only in its stimulatory role. Additional studies are required also in order to assess lipid autoxidation initiated by disruption of mitochondrial membranes (69) and its possible deteriorating effect upon the integrity of cytochrome oxidase.

Formation of Inactive Complex with Ferrocytochrome c—Although the observation of a first order reaction course for the aerobic oxidation of ferrocytochrome c in solution catalyzed by cytochrome oxidase (3, 70) has been confirmed repeatedly with all types of oxidase preparations (4, 9–12, 66) reaction data extending beyond 90% oxidation of the substrate have almost never been taken into consideration. Fig. 6 shows typical semi-logarithmic reaction plots obtained with the different oxidase samples under study here. Reactions were followed until they reached at least 99% completion. While the data for deoxycholate-treated heart muscle particles and oxidase-rich particles fit a straight line perfectly over the entire 99% range, deviations of different magnitude are seen with the Fowler and the Yonetani preparations. Similar reaction curves have been recorded with each of the preparations at initial substrate concentrations ranging from 4 to 70 \(\mu M\), and over at least a 10-fold variation of enzyme concentration. Only at extreme dilutions of the oxidase (requiring very long reaction times) was the effect not seen, since a gradual loss of active enzyme in the reaction mixture interferes with its observation. Linear and concave plots for treated oxidase-rich particles and Yonetani's preparation, respectively, were observed at pH values down to at least 5.8.

With the purified preparations, cytochrome c oxidation commences as a first order reaction; if this reaction were followed only until it is 60 to 80% complete, as is usually done, then a straight line fit to the data would be satisfactory. The departure from the original first order course at low remaining substrate concentrations is most pronounced with the Yonetani preparation. Since the effect is not found with solubilized particles (neither is it seen with particulate preparations of the Keilin-Hartree type as Minnaert's (4) rather complete reaction data show), it seems related to cytochrome oxidase purification. Several possible interpretations of this apparent activation phenomenon may be considered, based on (a) a slow increase in the number of active oxidase molecules after dilution in the reaction mixture, (b) differences in the affinities for ferri- and ferrocytochrome c, and (c) formation and dissociation of an inhibitory complex with substrate. The studies described below have shown the prevalence of the last of these alternatives with the purified preparation of Yonetani.

The oxidation-reduction of cytochrome c, measured at 550 nm, upon adding normally functioning oxidase to the system consisting of ascorbate, cytochrome c and \(O_2\), is expected to approach a steady state value exponentially with a rate constant equal to \(k_f + k_{asc}\), where \(k_f\) is the second order rate constant for reduc-
tion of ferriytochrome c by ascorbate. Fig. 7 presents a semilog plot the approach to the steady state observed with Yonetani's preparation. Under the conditions of this experiment, \( k_f = 0.032 \ \text{s}^{-1} \) (from the initial first order part of the reaction observed in a separate spectrophotometric run in the absence of ascorbate), \( k_r = 25 \ \text{m}^{-1} \ \text{s}^{-1} \), and \([\text{asc}] = 3.33 \times 10^{-3} \ \text{m} \). Thus \( k_f + k_r[\text{asc}] = 0.106 \ \text{s}^{-1} \) and we expect \( T_{1/2} = 6.5 \ \text{s} \). The results in Fig. 7, however, require fitting by two exponentials. The faster change, having about the expected \( T_{1/2} \) (6.9 s), should represent the response of cytochrome c oxidation-reduction to the faster change, having about the expected \( T_{1/2} \) (6.9 s), should require fitting by two exponentials. The slower phase (9.0 s) indicates a slow activation of a portion of inactive but appropriately disposed oxidase. In separate experiments we observed that this activation half time remained unchanged when using a 5-fold higher ascorbate concentration or at a 5-fold higher oxidase concentration, which suggests a spontaneous first order process. The relative amounts of instantaneously active and slowly activated enzyme may be calculated from an extrapolation of the slow phase to zero time; we find 70\% and 21\% of the total, respectively. But, since the ratio of the reaction rate to the remaining substrate concentration, \( v_f/|S|_{SO} \), more than doubles in the oxidation reaction in the absence of ascorbate (cf. Fig. 10, C), this type of activation must be rejected as the sole cause of the observed deviation from first order. This conclusion is confirmed by the data in Fig. 8. After completing a first oxidation of substrate, a new reaction initiated by adding ferriytochrome c again departs from first order, because of unequal coefficients \( k''S \) and \( k''P \) in the rate equation, should also be expressed in initial rate measurements.

Fig. 9 shows experimental \( |S|_{SO}/v_0 \) versus \( |S|_0 \) lines for the Yonetani preparation. Three initial product concentrations were used, namely 12.9, 30.4, and 42.9 \( \mu \text{m} \). Intersections with the \( |S|_0 \) axis are, 12.0, 27.5, and 43.5 \( \mu \text{m} \), respectively, below the crossing of the line for zero initial product concentration. Thus \( k''S/k''P \) appears to be very nearly 1, \( k''P = k''S \), which invalidates the proposed interpretation in terms of unequal affinities for ferro- and ferriytochrome c.

The deviation observed by Yonetani and Ray (10) resulting in convex semilogarithmic reaction plots at above neutral pH was also found with our preparation. In fact the combination of the two effects at pH 8.0 yielded semilog plots characterized by an inflection point. This supports our conclusion that an interpretation analogous to that of Yonetani and Ray (10) for deviation...
that a reversible unproductive binding with ferrocytochrome c. For rate measurements with added product, [S]o is the sum of the ferrous form contained in the sample of oxidized cytochrome c and the concentration of added reduced cytochrome c. Values of [P]o = [total cytochrome c] - [S]o are: 0 (O), 12.2 μM (H), 30.4 μM ( ), and 42.9 μM ( ). Theoretical lines are fitted by the method of least squares with the restriction that the lines for [P]o ≠ 0 be parallel to the one for [P]o = 0. Fe2+Cytc, ferrocytochrome c.

The third hypothesis that we considered relates increase in the v/[S] ratio with decreasing substrate concentration to a relief of inhibition by the dissociation of an inactive complex between the substrate and a fraction of the purified cytochrome aa3. For the purpose of concreteness let us take Minnaert’s Mechanism IV (Reaction 10) and assume a modification of the oxidase so that e’ means the total concentration of modified type of oxidase extending over about one reaction half time by [S]o, determined as follows. When no product is added, the total concentration of cytochrome c in the reaction mixture is taken as [S]o. For rate measurements with added product, [S]o is the sum of the ferrous form contained in the sample of oxidized cytochrome c and the concentration of added reduced cytochrome c. Values of [P]o = [total cytochrome c] - [S]o are: 0 (O), 12.2 μM (H), 30.4 μM ( ), and 42.9 μM ( ). Theoretical lines are fitted by the method of least squares with the restriction that the lines for [P]o ≠ 0 be parallel to the one for [P]o = 0. Fe2+Cytc, ferrocytochrome c.

Fig. 9. Plots of v/[S] as a function of [S]o at various [P]o with the Yonetani-type of oxidase. v is calculated by multiplying the rate constant for the initial apparent first order part of the reaction extending over about one reaction half time by [S]o, determined as follows. When no product is added, the total concentration of cytochrome c in the reaction mixture is taken as [S]o.

For rate measurements with added product, [S]o is the sum of the ferrous form contained in the sample of oxidized cytochrome c and the concentration of added reduced cytochrome c. Values of [P]o = [total cytochrome c] - [S]o are: 0 (O), 12.2 μM (H), 30.4 μM ( ), and 42.9 μM ( ). Theoretical lines are fitted by the method of least squares with the restriction that the lines for [P]o ≠ 0 be parallel to the one for [P]o = 0. Fe2+Cytc, ferrocytochrome c.

As for the aerobic oxidation of ferrocytochrome c (Fe2+Cytc, c) catalyzed by cytochrome oxidase purified by the method of Yonetani (O, 12.5 nM cytochrome aa3) and the method of Fowler et al. ( , 7.75 nM cytochrome aa3). The theoretical lines are derived from v/[S] with (v/[S])o = 0.0108 and 0.0445 s⁻¹, (v/[S])o = 0.0300 and 0.0588 s⁻¹, and [S]1/2 = 5 and 3.5 μM, for Yonetani’s and Fowler’s preparation, respectively.

The character of the sigmoidal curve is such that the midpoint shifts with the total concentration of cytochrome c. Initial rate measurements with substrate concentrations which are not exceedingly low therefore always refer to the relatively flat region at the right hand end of the curve. This explains why even at low substrate concentrations an initial apparent first order reaction is observed, and why the phenomenon does not become expressed by departure from linearity in the plots of Fig. 9.

The expression for (v/[S])o (Equation 14) is identical with that derived from mechanism IV for the first order rate constant of the normal uninhibited reaction (total enzyme concentration, e) (4). If it can be assumed that the rate constants for normal and anomalous oxidase in the preparation are the same then an estimate can be made of the relative amounts of enzyme in those two states from

\[
\frac{v}{[S]} \bigg| \text{S} = 0 \approx \frac{e' + e}{e'} \tag{16}
\]

In experiments with different initial ferrocytochrome c concentrations (13.5, 33.8, 47.4, and 67.7 μM) we found this ratio to be constant indicating that at least k-1/k+1 is unaffected by the modification that results in anomalous substrate binding. The value of the ratio (2.8) suggests the presence of about twice as much anomalous as normal oxidase in the final Yonetani preparation.

We note that k-1/k+1 = K_m using for the “uninhibited” oxidase (4). Therefore K_e may be calculated from the sigmoidal fit using Equation 15. We find 3.8 × 10⁻⁷ M for Yonetani’s enzyme, about 1 order of magnitude below the K_m.

The oxidase fractions at successive stages in the purification according to Yonetani were all found to exhibit qualitatively...
similar deviations from first order kinetics. The derived estimates of \( \frac{t_1}{[S]_0} \) and \( \frac{t_2}{[S]_0} \) are given in Table I. The results lead to the conclusion that the decline of MAO, max for the first fraction results entirely from the appearance in the preparation of oxidase molecules displaying a strong unproductive binding with ferrous cytochrome c. In subsequent fractions, however, the content of this anomalous oxidase appears to be inadequate to account completely for the decline of MAO, max.

\[ K_f \text{ for the Fowler preparation is found to be } 2.6 \times 10^{-7} \text{ M}. \]

Again, the amount of oxidase participating in the inhibitory combination with substrate appears not quite sufficient to explain the entire loss of MAO, max.

Serious difficulties hamper the investigation of the reaction course over a wider logarithmic range of substrate than presented in Fig. 10. Limitations arise on the one hand because of the shift of midpoint with \( t_1 \) which renders impossible the direct determination of \( \frac{t_1}{[S]_0} \), and on the other hand because of limitation of spectrophotometric sensitivity, contamination of the midpoints with \( \text{cyt c} \text{tot} \) which renders impossible the direct determination of \( \frac{t_1}{[S]_0} \), and on the other hand because of the shift of midpoint with \( t_1 \) which renders impossible the direct determination of \( \frac{t_1}{[S]_0} \), and on the other hand because of limited spectrophotometric sensitivity, contamination of the substrate with unreactive forms, and a possible departure from steady state in the catalytic process at very low remaining substrate concentrations. Accordingly, when the oxidase-catalyzed oxidation of 50 \( \mu \text{M} \) ferrocyanochrome c was followed up to 99.9\% complete with the aid of a sensitive slide wire on the Cary spectrophotometer, a marked slowing down was seen beyond the 99.5\% point. These experimental limitations suggest reservations about the proposed mechanism for activation and the quantitative conclusions derived from it.

**Blockade of Electron Transport between Cytochrome a and a3**

With an optical cell with 10-cm light path it is possible to observe spectrophotometrically the redox state of cytochrome oxidase under conditions comparable to those for activity determination with soluble cytochrome c. Because of the difficulty of mixing reagents quickly in a standard 10-cm cylindrical observation cell a system consisting of ascorbate, cytochrome c, oxidase and \( \text{O}_2 \) was chosen to obtain a steady state. Typical measurements of redox changes at 605 nm accompanying the transition from the aerobic steady state to the anaerobic state for deoxycholate-treated oxidase particles and Yonetani's enzyme preparation are compared in Table III. The results are strikingly different. While the oxidase of freshly solubilized particles is nearly completely oxidized in the steady state, the Yonetani enzyme has about half of its 605 nm band reduced. In fact, the oxidase derived from the particles behaves similarly to the components a and a3 of mitochondrial membrane oxidizing succinate (71, 72). On the other hand, our results with the purified enzyme confirm earlier extensive studies of the same system by Smith (8) and by Yonetani (50) who, from observations at 605 and 445 nm, concluded that cytochrome a alone is responsible for the partly reduced spectrum in the aerobic steady state.

Several investigators (8, 50, 73, 74) have shown an appreciable reduction of purified cytochrome oxidase in air by certain reducing agents, unaccompanied by significant oxygen uptake unless cytochrome c is added. Interestingly, the addition of cytochrome c did not yield a response of the redox state of the purified oxidase (8, 50). Based upon these results and similar ones of others using cytochrome c-deficient heart muscle particles (75, 76) objections have been raised against the classic linear reaction sequence (18) in the terminal part of the respiratory chain. Thus cytochrome a was placed on a side path (50, 74, 76), or a scheme was suggested that postulates an oxygenated oxidase whose decomposition depends on interaction with cytochrome c (73).

In these studies a homogeneous character for the oxidase preparations was assumed. The evidence described in previous sections and in Table III suggests heterogeneous properties for isolated oxidase. Because freshly solubilized active cytochrome oxidase is almost totally oxidized in the steady state, one may regard the partly reduced component present under similar conditions in Yonetani's enzyme as representing inactive oxidase (or slowly turning over, perhaps via intermolecular electron transfer to active units), in which the transfer from a11 to a111 is blocked due to either a failure of cytochrome c to interact properly with the a1 complex or to some structural disjunction at the a-a3 site. The presence of this inactive species provides a simple explanation for the stability towards \( \text{O}_2 \), for partly reduced absorption bands and for lack of change in redox state when oxygen consumption is initiated by adding cytochrome c. The often quoted "dynamic" evidence for the existence of a and a3 components in cytochrome oxidase, namely the difference in the extent of reduction of the 605 and 445 nm bands during the aerobic steady state observed with a purified preparation (8), may actually reflect the properties of an inactive portion in the preparation. Considering the relative contribution of cytochrome a to the band at 605 nm (19, 77) the results with Yonetani's preparation (Table III, and Ref. 19) suggest that the catalytically inactive species responsible for the reduced cytochrome a in the aerobic steady state amounts to 70\% or more of the total preparation.

A blockade of electron transport between (some of the) a and a3 components of purified cytochrome oxidase is also evident from stopped flow kinetic measurements on the reaction with reduced cytochrome c, where a fast reduction involving a is followed by a much slower electron transfer to other accepting centers (78, 79). The existence of a complex between a11 and c111 that is inhibitory to the reduction of a3111 was suggested by the former group of workers (78), while the latter ascribed novel spectral properties to cytochrome a and concluded that the rate constants of the slow reaction centers are affected by \( \text{O}_2 \). In our view, the reaction course with ferrocyanochrome c observed at 605 and 445 nm largely reflects the inactive or latent portion of the purified oxidase as evidenced by the relatively small influence of aerobic conditions on the extent of reduction reached after the fast initial phase, and by the finding of a low molecular activity. The joint reduction of some active aa3 and inactive a may explain the unusual difference spectrum ascribed to cytochrome a, and since the slow reacting centers belong to catalytically inactive

---

TABLE III

<table>
<thead>
<tr>
<th>Oxidase sample</th>
<th>( \Delta A ) at 605 nm (anaerobic state minus aerobic steady state)*</th>
<th>( \Delta A ) at 605 nm reduced minus oxidized cytochrome oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycholate-treated oxidase-rich particles</td>
<td>0.0122, 0.0129</td>
<td>0.0128</td>
</tr>
<tr>
<td>Yonetani's preparation</td>
<td>0.0005, 0.0051</td>
<td>0.0128</td>
</tr>
</tbody>
</table>

* Corrected for contribution from cytochrome c, 10-cm cell.
oxidase in the preparation, there appears to be no need for an influence of O₂ on their rate constants. On the other hand, the proposed inhibitory complex formation with cytochrome c is similar to the conclusion derived from steady state kinetics in the previous section (cf. Reaction 11 where ES' could represent proposed inhibitory complex formation with cytochrome c is inhibitor, nor the removal of phospholipid per se, seems to influence of O₂ on their rate constants. On the other hand, the treatment, may be ascribed most readily to transformation of u-u₃ interaction, and/or the potential for activation by SDS characteristics such as inhibitory complexation with substrate, disabled oxidase in the preparation, there appears to be no need for an inactive form(s) indicate a sub-

CONCLUSION

The decline of molecular activity and the appearance in the course of cytochrome oxidase purification of anomalous characteristics such as inhibitory complexation with substrate, disabled a-a₃ interaction, and/or the potential for activation by SDS treatment, may be ascribed most readily to transformation of u-u₃ interaction, and/or the potential for activation by SDS characteristics such as inhibitory complexation with substrate, disabled oxidase in the preparation, there appears to be no need for an inactive form(s) indicate a sub-

Acknowledgements—We are greatly indebted to Mr. Leonard Evans for skillful assistance in several aspects of this work, and to Dr. Joann S. Loehr for her invaluable help with the polyacrylamide gel electrophoresis. We thank Professor Allan Davison for numerous discussions which greatly contributed to this study.

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