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CYTOCHROME OXIDASE FROM *PSEUDOMONAS AERUGINOSA*

II. REACTION WITH COPPER PROTEIN

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SUMMARY

The reaction between a cytochrome oxidase and a copper protein from *Pseudomonas aeruginosa* has been studied by a rapid mixing technique. The data support the view that a complex is formed rapidly between the two proteins and is followed by a transfer of electrons in either direction. Reduced copper protein donates an electron to the heme *c* moiety of the oxidase with an apparent first-order rate constant of about 30 s^{-1} while the transfer in the reverse direction proceeds with a constant of about 120 s^{-1}. The reaction between the copper protein and the heme *c* of the oxidase is followed by a much slower internal reaction involving electron transfer between the heme *c* and heme *d*.

The kinetic data have been analyzed in terms of the thermodynamics of the interactions. This analysis indicates that the copper protein has a ΔE of about 0.038 V more positive than the heme *c* component, a value that compares favorably with that of 0.040 V obtained by equilibrium methods. The value of ΔE obtained by the kinetic method for the internal reaction is less precise but is reasonably close to that of 0.070 V determined by an equilibrium technique.

INTRODUCTION

Pseudomonas cytochrome oxidase (ferrocytochrome c_2:O_2 oxidoreductase, EC 1.9.3.2) functions in the terminal respiration of cells of *Pseudomonas aeruginosa*[1–6]. The oxidase can donate electrons either to molecular oxygen[3,4,12] or to nitrite[5,7,8] although it appears that *in situ* it functions with nitrite rather than oxygen, since *Pseudomonas* cytochrome oxidase is only synthesized when the cells are cultured anaerobically in the presence of nitrate[9]. Thus, it might be described more appropriately as a nitrite reductase.

Pseudomonas cytochrome oxidase has been purified to a state of electrophoretic homogeneity as a water-soluble protein containing one heme *c* moiety and one heme *d* group per molecular weight of 115000[10]. The purified preparation has been found to oxidize a *c*-type cytochrome (*c*-551)[11,12] as well as a copper protein of the blue type[13,14], both of which are synthesized by the organism[1,10].

In an effort to further elucidate the enzymic mechanims of terminal electron transfer we have investigated the reactions in which *Pseudomonas* cytochrome

oxidase participates. In this communication we shall describe details of the reaction between the oxidase and the *Pseudomonas* copper protein.

EXPERIMENTAL

Materials

Pseudomonas cytochrome oxidase and *Pseudomonas* copper protein were purified by the method of Gudat *et al.*[10]. The concentration of heme *c* in *Pseudomonas* cytochrome oxidase is based on a value of ε (549 nm) of 30.2 $mM^{-1} \cdot cm^{-1}$ (ref. 4) after reduction with a few grains of sodium dithionite. The concentration of heme *d* in *Pseudomonas* cytochrome oxidase was calculated from ε (620 nm) = 24 $mM^{-1} \cdot cm^{-1}$ (ref. 15) for the reduced pyridine hemochrome. The concentration of copper protein is based on a value of ε (630 nm) of 6.95 $mM^{-1} \cdot cm^{-1}$ (ref. 14) with the copper in the cupric state.

All reagents unless specified otherwise were of analytical reagent grade. Sodium dithionite was obtained from Hardman and Holden, Miles Platting, Manchester, England. Ascorbic acid was of U.S.P. grade from Merck and Company; it was usually employed as a solution of the potassium salt. Carbon monoxide (C.P.) was purchased from Matheson.

Methods

The stopped flow and associated equipment have been described previously[16,17]. The apparatus was employed on-line with a Digital Equipment Corporation Model PDP-8I digital computer and A/D converter. The computer was programmed for flexible data acquisition to select points on the reaction curve, smooth them by a least-squares procedure, and to print these as Δabsorbance. Furthermore, the computer was used simultaneously to calculate and print the first-order rate constants of the reaction, both cumulative and point to point.

Solutions containing the reactants were made anaerobic in a tonometer by several cycles of evacuation and flushing with oxygen-free nitrogen. The tonometer was left with a slight positive pressure of nitrogen. The reactant was reduced by titration with anaerobic reducing agent (usually 0.1 M ascorbate) by means of a gas-tight microsyringe inserted through a rubber serum cap near the upper end of the tonometer. The reduction of the reactant was monitored in a Beckman Model DK-2A spectrophotometer by means of a cuvette fitted to the lower end of the tonometer. The titration was terminated just before complete reduction was reached and the reactant was then transferred anaerobically to the stopped-flow apparatus. For experiments in the presence of CO the tonometer was evacuated after reduction of the oxidase and filled with 1 atm of CO. In these experiments solutions of the copper protein also contained 1 atm of CO.

Because of an overlap in the optical absorption spectra of the reactants it was necessary to apply corrections at the wavelengths used to monitor the reactions. For the contribution of the copper protein at 549 nm (the maximum of the heme *c* group) the following correction factor was applied:

$$\Delta A_{549\,nm} = 0.35 \cdot \Delta A_{595\,nm}$$

For the contribution of the heme *c* component at 460 nm (the maximum of the heme *d* group) the following correction factor was used:

$$\Delta A_{460\,nm} = 0.465 \cdot \Delta A_{549\,nm}$$

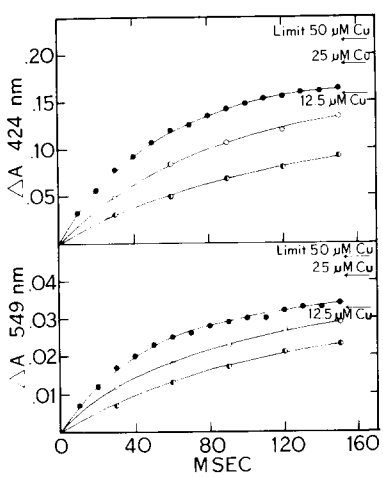
It was found unnecessary to make any corrections for absorbance changes at 595 nm (the wavelength used to monitor changes in the copper protein) since this was an isosbestic point for the components of the oxidase. Furthermore, no correction was required at 424 nm* since only the heme *c* was found to be involved in absorbance changes at that wavelength.

RESULTS

Reaction of oxidized Pseudomonas cytochrome oxidase with reduced copper protein

The mixing of reduced copper protein with oxidized *Pseudomonas* cytochrome oxidase under anaerobic conditions produces biphasic absorbance changes at 595, 549, and 424 nm and a monophasic change at 460 nm. As seen in Fig. 1 the magnitude and rate of the absorbance changes are affected by the ratio of copper protein to oxidase. Thus, as the concentration of copper protein is increased the rate as well as the total absorbance change increases.

The biphasic character of the absorbance changes is illustrated in Fig. 2 where



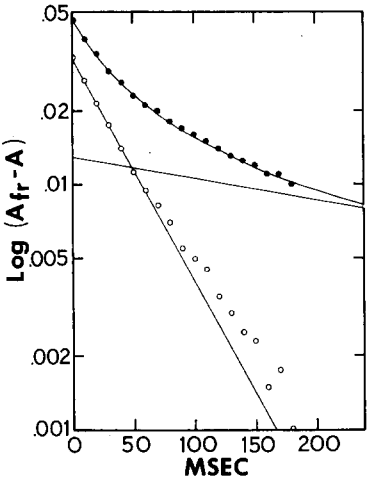


Fig. 1. Reduction of *Pseudomonas* cytochrome oxidase (15 μM) by reduced copper protein followed in the stopped-flow apparatus at 424 and 549 nm. Temperature was 20 °C; buffer was 0.1 M phosphate, pH 6.6. ●, 50 μM reduced copper protein; ○, 25 μM reduced copper protein; ◐, 12.5 μM reduced copper protein. The final changes in absorbance are indicated. Path length, 2 cm. Concentrations given are those before mixing.

Fig. 2. Reduction of *Pseudomonas* cytochrome oxidase (15 μM) by 50 μM copper protein followed at 549 nm. Temperature was 20 °C; buffer was 0.1 M phosphate, pH 6.6. ●, data points from the reaction trace; ○, points obtained after subtracting the slow phase of the reaction (solid line) from the points of the reaction trace.

* This wavelength was chosen because insufficient light was transmitted at the absorbance maximum at 418 nm with the concentrations of oxidase used in these experiments.

the logarithm of the absorbance changes is plotted as a function of time. The line represented by the solid circles is curved because it contains the elements of two reactions, one rapid and the other slower. If the elements of the slower reaction (indicated by the solid line) are subtracted from the composite curve then a straight line (indicated by the open circles) is obtained which represents the rapid phase of the reaction. In the reaction illustrated in Fig. 2, where 50 μM copper protein was mixed with 15 μM *Pseudomonas* cytochrome oxidase, the rapid phase has a half-time of 32 ms and is virtually complete within 200 ms. The slower phase, on the other hand, has a half-time of at least 200 ms and requires several seconds to reach completion.

The absorbance changes at 549 nm and 424 nm reflect changes in the oxidation state of the heme *c* moiety of *Pseudomonas* cytochrome oxidase; the changes at 460 nm represent a change in the state of the heme *d* component; those at 595 nm are due to changes in the oxidation state of the copper protein. Analysis of the data indicates that the rapid portion of the biphasic reaction represents the reduction of the heme *c* component of the oxidase by the copper protein. Thus, as shown in Fig. 3, when the absorbance changes at 549 nm are plotted against the changes at 424 nm, straight lines are obtained with a slope, after correction for the contribution of the copper protein at 549 nm, of 4.6. This value is essentially the same as the ratio obtained from static absorption spectra for the heme *c* component of the oxidase. When the absorbance changes at 549 nm are plotted against the changes at 595 nm, straight lines are obtained with a slope of 2.8. This value is similar to the ratio of the $\Delta\varepsilon_{549\ nm}$ (c^{2+} *minus* c^{3+}):$\Delta\varepsilon_{595\ nm}$ (Cu^{1+} *minus* Cu^{2+}).

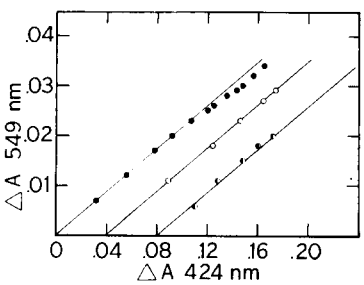


Fig. 3. The relation between the absorbance changes at 549 nm and those at 424 nm from the data of Fig. 1. Symbols are the same as in Fig. 1. The scales for $\Delta A_{424\ nm}$ are displaced for clarity.

The total absorbance changes at 460 nm are rather small due to the fact that the heme *d* group has a more negative oxidation–reduction potential than either the heme *c* group or the copper protein and because the heme *c* is contributing to the absorbance change in the opposite direction. As a result the signal-to-noise ratio of the data obtained in these experiments at 460 nm is not particularly good and precise absorbance results are difficult to attain. It seems, however, that on the basis of an approximation, the absorbance changes are monophasic. Moreover, it would appear that their rates are comparable to the slower phase of the biphasic reactions observed for the heme *c* and the copper protein with a half-time of approximately 500 ms.

Reaction of oxidized copper protein with reduced Pseudomonas cytochrome oxidase

In addition to serving as a donor of electrons to *Pseudomonas* cytochrome

oxidase the copper protein can also accept electrons from the oxidase. Thus, the addition of oxidized copper protein to reduced *Pseudomonas* cytochrome oxidase under anaerobic conditions results in biphasic absorbance changes at 595, 549, and 424 nm and a monophasic change at 460 nm. As in the case of the transfer of electrons from reduced copper protein to the oxidase the rate and magnitude of the absorbance changes are affected by the ratio of copper protein to oxidase. Fig. 4 shows the absorbance changes that occur at 549 and 424 nm after mixing *Pseudomonas* cytochrome oxidase of a fixed concentration with copper protein at three different concentrations.

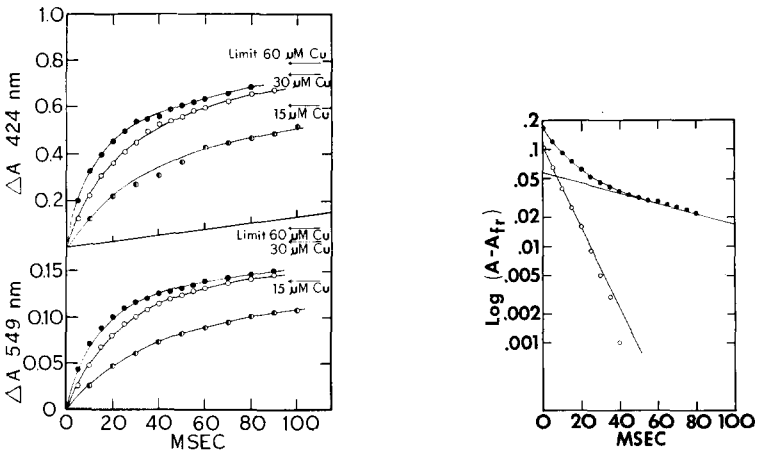
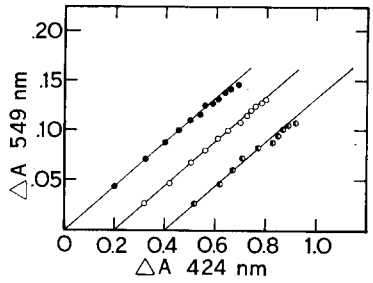


Fig. 4. Reduction of copper protein by reduced *Pseudomonas* cytochrome oxidase (15 μM) in the stopped flow apparatus at 424 and 549 nm. Temperature was 20 °C; buffer was 0.1 M phosphate, pH 6.6. ●, 60 μM oxidized copper protein; ○, 30 μM oxidized copper protein; ◐, 15 μM oxidized copper protein. The final changes in absorbance are indicated. Path length, 2 cm. Concentrations given are those before mixing. The base line for the changes at 424 nm is displaced to accommodate the figures for the limits of the changes at 549 nm.

Fig. 5. Reduction of copper protein (60 μM) by 15 μM (each heme group) *Pseudomonas* cytochrome oxidase followed at 549 nm. Temperature was 20 °C; buffer was 0.1 M phosphate, pH 6.6. ●, data points from the reaction trace; ○, points obtained after subtracting the slow phase of the reaction (solid line) from the points of the reaction trace.

When the data in Fig. 4 are plotted logarithmically as a function of time a curved line is obtained as shown in Fig. 5. This curved line contains the elements of two reactions, one rapid and the other slower. These reactions can be separated and plotted as two straight lines representative of pseudo-first-order reactions. In the case where the highest concentration of copper protein (60 μM) was mixed with 15 μM *Pseudomonas* cytochrome oxidase the rapid phase was calculated to have a half-time of 8 ms and was virtually complete within 50 ms. On the other hand, the slower phase was observed to have a half-time of 75 ms and required several seconds to reach completion. Analysis of these data indicates that the rapid portion of the reaction represents the oxidation of the heme *c* component of the oxidase by the copper protein. Thus, as seen in Fig. 6 the absorbance changes at 549 nm plotted against those at 424 nm result in straight lines that have a slope, after correcting for the contribution of



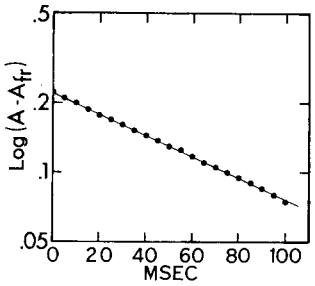


Fig. 6. The relation between the absorbance changes at 549 nm and those at 424 nm from the data of Fig. 4. Symbols are the same as in Fig. 4. The scales for $\Delta A_{424\text{ nm}}$ are displaced for clarity.

Fig. 7. Reduction of copper protein (60 μM) by 15 μM (each heme group) *Pseudomonas* cytochrome oxidase followed at 460 nm. Temperature was 20 °C; buffer was 0.1 M phosphate, pH 6.6.

copper protein at 549 nm, of about 4.5. Furthermore, the absorbance changes at 549 nm, when plotted against those at 595 nm, result in straight lines with a slope of about 3.

The absorbance changes at 460 nm are much greater in these experiments than in those where reduced copper protein initiated the reaction. This result occurs because the oxidation–reduction potentials of the reactants favor practically complete oxidation of the heme *d* which is represented by the absorbance changes at 460 nm. When appropriate corrections are made for the contribution of heme *c* the absorbance changes at 460 nm, as seen in Fig. 7, are monophasic and have a half-time of about 70 ms. These changes, when plotted against the slow absorbance changes at 460 nm, result in a proportional relationship. There appears to be little, if any, effect on the rate of the slow changes on varying ratios of copper protein and oxidase.

Reaction of oxidized copper protein with reduced Pseudomonas cytochrome oxidase inhibited by CO

CO combines with the reduced heme *d* component of *Pseudomonas* cytochrome oxidase but not with the heme *c* moiety[3,10]. When oxidized copper protein is mixed with reduced oxidase in the presence of CO there is no change in absorbance at 460 nm after correcting for the contribution of the heme *c* component. Moreover, the absorbance changes at 595, 549, and 424 nm are essentially monophasic. Thus, when the absorbance changes at 549 and 424 nm are plotted logarithmically as a function of time there is a straight line for each as seen in Fig. 8. The half-time

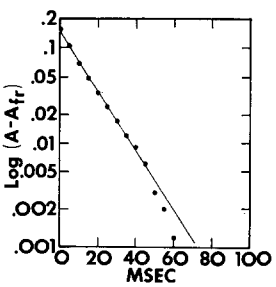


Fig. 8. Reduction of copper protein (60 μM) by 15 μM (each heme group) *Pseudomonas* cytochrome oxidase followed at 549 nm in the presence of 1 atmosphere of carbon monoxide. Temperature was 20 °C; buffer was 0.1 M phosphate, pH 6.6.

for the reaction where 60 μM copper protein was mixed with 15 μM of *Pseudomonas* cytochrome oxidase is about 7 ms, a value that compares favorably with the rapid phase of the uninhibited biphasic reaction. In these reactions the rate is also affected by the ratio of copper protein and oxidase with the half-times corresponding to the half-times of the rapid phase of the biphasic reactions.

DISCUSSION

The addition of reduced copper protein to oxidized *Pseudomonas* cytochrome oxidase produces a rapid reduction of the heme *c* component of the oxidase and a slower reduction of the heme *d* moiety. Furthermore, the reaction of the reduced *Pseudomonas* cytochrome oxidase with oxidized copper protein results in a rapid oxidation of the heme *c* moiety and a slower oxidation of the heme *d*. We interpret these results to indicate that the copper protein interacts with the *Pseudomonas* cytochrome oxidase and that a rapid transfer of an electron occurs between the copper and the heme *c*. The rapid transfer is followed by a slower transfer of an electron between the heme *d* and the heme *c* components of the oxidase. This interpretation is based on several considerations. First, the ratio of the changes in oxidation state of the copper protein and the heme *c* of the oxidase agree closely with the ratio predicted from the extinction coefficients of these electron carriers and indicates an equivalent transfer of electrons. Second, the rapid changes that occur between reduced *Pseudomonas* cytochrome oxidase and oxidized copper protein are duplicated in the presence of CO; under these conditions the oxidation of heme *d* is blocked and presumably only the heme *c* is able to donate an electron. Finally, the ratio of the slow changes in the oxidation states of the heme *c* and heme *d* agree closely with the ratio predicted for the extinction coefficient of these components.

The rates of the reactions involving heme *d* are at least an order of magnitude less than those involving heme *c* and the copper protein. The rates of the changes in the oxidation state of the copper protein and the heme *c* of the oxidase are altered by changes in their relative concentrations. Thus, when the concentration of the copper protein is increased with respect to the oxidase there is a significantly higher rate of electron transfer. If the reciprocals of the observed pseudo first order rates of both the forward and reverse transfers are plotted as a function of the reciprocal of the copper protein concentration, a limit of the first-order rate constants for each transfer can be estimated (see Fig. 9). These values are 29 s^{-1} for the transfer between reduced copper protein and oxidized heme *c* and 120 s^{-1} for the transfer between reduced heme *c* and oxidized copper protein. The hyperbolic character of the observed rate constants when plotted as a function of copper concentration suggests that a complex is formed between the oxidase and the copper protein[18]. Furthermore, the data indicate that the rate of complex formation is considerably faster than the rate of electron transfer since little if any lag in absorbance changes was observed after mixing the reactants (see Figs 2 and 5). These results suggest that the rate of the reaction between the oxidase and the copper protein is close to that expected for a diffusion-controlled reaction between molecules of this size (see Alberty and Hammes[19]). In addition, rates of reactions between oxidation–reduction proteins that are even higher than those observed here have been found for the reaction of beef heart cytochrome oxidase and cytochrome *c*[20] and of yeast cytochrome *c* peroxidase and cytochrome *c*[21].

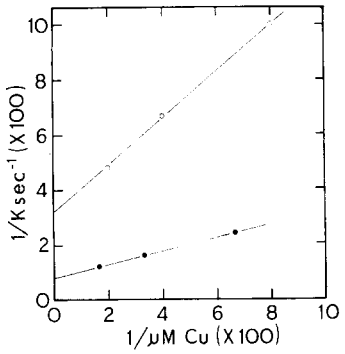


Fig. 9. Double-reciprocal plot of pseudo-first-order rate constants as a function of copper protein concentrations for the reaction with *Pseudomonas* cytochrome oxidase (15 μM). ○, reaction between reduced copper protein and oxidized oxidase; ●, reaction between oxidized copper protein and reduced oxidase. The intercept at the abscissa gives the limit rate constants for each reaction.

The overall reaction involving *Pseudomonas* cytochrome oxidase and the copper protein may be expressed as

$$Cu^{1+} + c^{3+}d^{3+} \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightleftharpoons Cu^{2+} + c^{2+}d^{3+} \tag{1}$$

$$c^{2+}d^{3+} \rightleftharpoons c^{3+}d^{2+} \tag{2}$$

$$Cu^{1+} + c^{3+}d^{2+} \rightleftharpoons ES_1' \rightleftharpoons ES_2' \rightleftharpoons Cu^{2+} + c^{2+}d^{2+} \tag{3}$$

We have already mentioned that the portion of the reaction involving a transfer of electrons to or from heme *d* is approximately an order of magnitude slower than the respective oxidation or reduction of the heme *c* group by the copper protein. Thus, the contribution of heme *d* to the rates of the rapid reactions involving heme *c* and the copper protein can be disregarded without significantly affecting the resulting rate constants. As a result the reactions expressed in equations 1, 2, and 3 can be simplified to

$$Cu^{1+} + c^{3+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Cu^{2+} + c^{2+} \tag{4}$$

It is of interest to compare the kinetic results obtained here with thermodynamic data reported by Horio *et al.*[22] and Kamen and Horio[23]. In order to evaluate the data in this way and to determine the overall equilibrium constant, it is necessary to determine Ks_1 and Ks_2 which are defined as the association constants for the formation of ES_1 and ES_2, respectively, from the reactants on the opposite sides of the equation. From our data we calculate Ks_1 and Ks_2 to be both equal to $2.6 \cdot 10^{-5}$ M. Since these constants are equal they cancel out one another in the calculation of the equilibrium constant. Thus, the equilibrium constant for the reaction is equal to 0.242. In order to compare this value with the value for the difference in the oxidation–reduction potentials, ΔE, between the heme *c* component of the oxidase and the copper protein,

where $\Delta E = E_h$ (heme *c*) $- E_h$ (copper protein), the Nernst equation must be re-written as

$$\Delta E = \frac{RT \ln K_{eq}}{nF} \tag{5}$$

Using K_{eq} equal to 0.242 we have calculated ΔE to be -0.036 V. This value is remarkably close to that obtained by subtracting the value of $E_h = 0.328$ V, obtained by workers in Okunuki's laboratory[6], for copper protein from the value of $E_h = 0.288$ V, reported by Kamen and Horio[23], for the heme *c* moiety of the oxidase.

It is considerably more difficult to evaluate the slow changes in the reaction between *Pseudomonas* cytochrome oxidase and the copper protein in order to compare them with existing thermodynamic data. The chief difficulty stems from the small absorbance changes and low signal to noise ratios observed during the slow phase when reduced copper protein is mixed with oxidized oxidase. Thus, the first-order rate constants calculated for reactions of the latter type are probably correct only to an order of magnitude. For this reason only an approximate comparison can be made with the thermodynamic data.

The equation for the slow phase of the reaction can be expressed as

$$c^{2+} + d^{3+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} c^{3+} + d^{2+} \tag{6}$$

where *c* and *d* are the heme *c* and heme *d* groups, respectively, of *Pseudomonas* cytochrome oxidase. With the present data it is probably safe to set limits for k_1 of 0.2 to 2.0 s^{-1}. The data for the reverse reaction are stronger, because of greater absorbance changes, and k_{-1} is equal to about 10 s^{-1}. With these limits K_{eq} for the reaction is equal to between 0.02 and 0.20 and the value for ΔE is between -0.040 and -0.100 V, with the heme *d* group having a more negative mid-point potential than the heme *c* moiety. These values are not inconsistent with the ΔE of -0.070 V reported by Horio *et al.*[22] using a thermodynamic method. Thus, although our current data on the slow phase of the reaction between *Pseudomonas* cytochrome oxidase and the copper protein are not ideal they are not out of line with the thermodynamic findings.

The observation that the copper protein, which is not autooxidized and is normally thought of as a donor of electrons to *Pseudomonas* cytochrome oxidase, has a more positive mid-point potential than does either the heme *c* or heme *d* components of the oxidase raises many interesting questions not the least of which is its possible role in controlling the electron transfer reaction. A considerable excess of reduced copper protein, vis à vis *Pseudomonas* cytochrome oxidase, would have to be present in order to donate electrons *via* the oxidase to a terminal electron acceptor at an optimal rate. Thus, substrate would be oxidized significantly only when its concentration tended to become quite high. Conversely, a lower concentration of substrate would result in a considerable turning down of the overall process.

Whether cytochrome *c*-551, another electron donor of the oxidase, acts similarly to the copper protein is unknown at this time. This cytochrome has been reported to have a mid-point potential of $+0.286$ V[6] which places it very close thermodynamically to the heme *c* component of the oxidase. In addition, Antonini and his collaborators[24]

found that cytochrome *c*-551 interacts readily with the copper protein and that electrons are transferred between the two proteins at rates consistent with those observed here for the oxidase and the copper protein. Unfortunately, the close overlap of the absorption spectra of the cytochrome *c*-551 and the heme *c* moiety of the oxidase precludes a duplication of the rapid-mixing experiments with the copper protein that we have reported here. One experimental solution to this problem may be to freeze the reactants at stepwise intervals after mixing and quantitatively measure the electron paramagnetic resonance signals of the heme iron[10].

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