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CYTOCHROME OXIDASE FROM *PSEUDOMONAS AERUGINOSA* IV. REACTION WITH OXYGEN AND CARBON MONOXIDE

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SUMMARY

The reaction between a cytochrome oxidase from *Pseudomonas aeruginosa* and oxygen has been studied by a rapid mixing technique. The data indicate that the heme d_1 moiety of the ascorbate-reduced enzyme is oxidized faster than the heme *c* component. The oxidation of heme d_1 is accurately second order with respect to oxygen and has a rate constant of $5.7 \cdot 10^4\ M^{-1} \cdot s^{-1}$ at 20 °C. The oxidation of the heme *c* has a first-order rate constant of about $8\ s^{-1}$ at infinite concentration of O_2. The results indicate that the rate-limiting step is the internal transfer of electrons from heme *c* to heme d_1. These more rapid reactions are followed by more complicated but smaller absorbance changes whose origin is still not clear.

The reaction of ascorbate-reduced oxidase with CO has also been studied and is second order with a rate constant of $1.8 \cdot 10^4\ M^{-1} \cdot s^{-1}$. The initial reaction with CO is followed by a slower reaction of significantly less magnitude. The equilibrium constant for the reaction with CO, calculated as a dissociation constant from titrimetric experiments with dithionite-reduced oxidase, is about $2.3 \cdot 10^{-6}$ M. From these data a rate constant of $0.041\ s^{-1}$ can be calculated for the dissociation of CO from the enzyme.

INTRODUCTION

Pseudomonas cytochrome oxidase (ferrocytochrome c_{551} : oxidoreductase, EC 1.9.3.2) is a water-soluble enzyme that can be purified to homogeneity from extracts of *Pseudomonas aeruginosa* [1–3]. The purified enzyme has a molecular weight of about 120 000 and consists of two equivalent subunits [2, 3]. The dimeric form appears to contain four heme groups, of which two are heme *c* and two are heme d_1 [4]. Although *Pseudomonas* cytochrome oxidase probably functions in vivo as a nitrite reductase [5], it does undergo oxidation by molecular oxygen [6, 7]. Okunuki and his collaborators [8, 9] have shown that the enzyme is inhibited by cyanide and CO [8] and that under certain conditions the products of the oxidation are oxidized enzyme and either H_2O_2 or presumably water [9]. Because of these properties *Pseudomonas* cyto-

chrome oxidase is an interesting model for comparison with the detergent-solubilized mitochondrial cytochrome *c* oxidase.

We have previously reported studies of the interaction between *Pseudomonas* cytochrome oxidase and one of its electron donors, the blue copper protein (azurin) [10]. In that study we reported that electrons from azurin are transferred first to the heme *c* moiety of the oxidase and that subsequently there occurs a slower internal transfer of electrons to heme d_1. In the current study we show that when *Pseudomonas* cytochrome oxidase in the reduced form interacts with O_2, the heme d_1 becomes oxidized at a rate faster than the heme *c*. We also present some data on the reaction between the reduced *Pseudomonas* cytochrome oxidase and CO.

EXPERIMENTAL

Materials

Pseudomonas cytochrome oxidase was purified to electrophoretic homogeneity by the method of Gudat et al. [3]. The concentration of heme *c* in *Pseudomonas* cytochrome oxidase is based on a value of ε (549 nm) of 30.2 $mM^{-1} \cdot cm^{-1}$ [1] as reduced with a few grains of $Na_2S_2O_4$. The concentration of heme d_1 in *Pseudomonas* cytochrome oxidase was calculated from ε (620 nm) of 24 $mM^{-1} \cdot cm^{-1}$ [11] for the dithionite-reduced pyridine hemochrome.

All reagents unless specified otherwise were of analytical reagent grade. $Na_2S_2O_4$ was obtained from Hardman and Holdman, Miles Platting, Manchester, U.K. Ascorbic acid was of U.S.P. grade from Merck and Co.; it was usually employed as a solution of the potassium salt. CO was purchased from Matheson as the C.P. grade.

Methods

The stopped-flow and associated equipment have been described previously [12, 13]. The apparatus was employed on-line with a Digital Equipment Corporation model PDP-8I digital computer and A/D converter as in our previous report [10].

Anaerobic and reduced solutions of the enzyme were obtained and handled as before [10]. The correction for absorbance changes at 460 nm due to a contribution of heme *c* was the same as that used earlier [10]. The following correction factor was used at 645 and 660 nm to allow for the contribution of heme *c* in this region:

$$\Delta A_{645\,nm} = \Delta A_{660\,nm} = 0.027 \cdot \Delta A_{549\,nm}$$

For the titration experiments with CO, 3.0 ml of the enzyme solution (about 35 μM) in 0.05 M potassium phosphate (pH 6.6) was reduced with a few grains of $Na_2S_2O_4$ and transferred by means of a syringe to an anaerobic cuvette fitted with a rubber serum cap. In order to monitor absorbance changes two identical anaerobic cuvettes containing equivalent concentrations of reduced *Pseudomonas* cytochrome oxidase were placed in the sample chamber of a Beckman model DK-2A recording spectrophotometer at 25 °C. One cuvette was placed in the reference beam and the other in the sample beam. 10-μl aliquots of 0.05 M potassium phosphate (pH 6.6) equilibrated with one atmosphere of CO at 25 °C were added to one of the cuvettes in the spectrophotometer by means of a gas-tight microsyringe inserted through the serum cap. An identical volume of anaerobic buffer equilibrated with one atmosphere

of N_2 was added to the second cuvette in order to allow for absorbance changes due to dilution. After mixing and waiting for 1 min, the absorption spectrum in the visible region was recorded.

RESULTS

Difference spectrum of Pseudomonas cytochrome oxidase

In order to clarify the significance of the absorbance changes obtained by stopped flow spectrophotometry the difference spectrum (reduced vs. oxidized) of the *Pseudomonas* cytochrome oxidase is illustrated in Fig. 1. The absorption maxima at 660 and 460 nm represent the α- and Soret-bands, respectively, of the heme d_1 component of the enzyme while those at 552–549, 521 and 418 nm represent the α-, β- and Soret-bands, respectively, of the heme *c* moiety. The trough at about 645 nm is due to the α-band of oxidized heme d_1.

Reaction of reduced Pseudomonas cytochrome oxidase with oxygen

The reaction of ascorbate-reduced *Pseudomonas* cytochrome oxidase with O_2

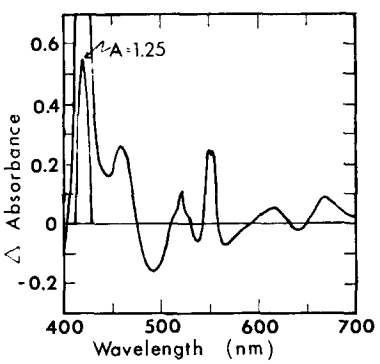

Fig. 1. Difference spectrum (reduced vs. oxidized) of *Pseudomonas* cytochrome oxidase. The concentration of enzyme was 14.5 μM (expressed as heme *c*) and was dissolved in 0.05 M potassium phosphate (pH 6.6). The enzyme was reduced with a few grains of $Na_2S_2O_4$.

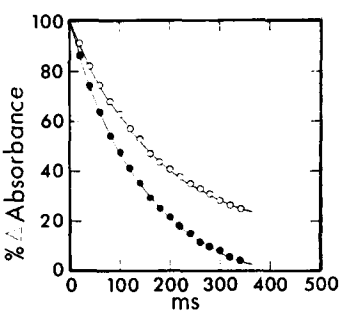

Fig. 2. Reaction of reduced *Pseudomonas* cytochrome oxidase with O_2 followed in the stopped-flow apparatus at 460 nm (●) and at 549 nm (○). The concentration of enzyme was 12 μM (expressed as heme *c*) and that of O_2, 142 μM, after mixing. Other conditions were: temperature, 20 °C; 0.05 M potassium phosphate (pH 6.6); light path, 2 cm,

was examined systematically by stopped-flow spectrophotometry over a range of wavelengths and at several concentrations of O_2. Fig. 2 shows the reaction of 142 μM O_2 with 12 μM *Pseudomonas* cytochrome oxidase. The absorbance change takes place more rapidly at 460 nm, the Soret-band of the heme d_1 moiety, than at 549 nm, the α-band of the heme *c* component. The change at 460 nm proceeds with a half-time of about 85 ms and is nearly complete at 400 ms after mixing. The change at 549 nm, on the other hand, proceeds with a half-time of about 150 ms and at 400 ms after mixing the solution still contains a significant amount of unreacted material.

At lower initial concentrations of O_2 the rate of change at both 460 and 549 nm is slower. For example, in the presence of 71 μM O_2 and 12 μM *Pseudomonas* cytochrome oxidase the change at 460 nm proceeds with a half-time of about 175 ms while the half-time of the change at 549 nm is about 260 ms. If the pseudo-first-order rate constants for the changes at 460 nm are plotted as a function of O_2 concentration a straight line is obtained as illustrated in Fig. 3. This result indicates that the reaction of the heme d_1 moiety is second order with respect to the concentration of O_2. The second-order rate constant calculated from these data is approx. $5.7 \cdot 10^4$ $M^{-1} \cdot s^{-1}$.

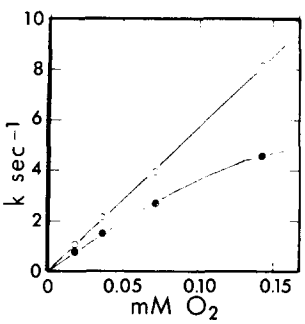

Fig. 3. Relationship between the measured pseudo-first-order rate constants for the oxidation of the heme d_1 (○) and heme *c* (●) moieties of *Pseudomonas* cytochrome oxidase and the concentration of O_2. Conditions were the same as described in Fig. 2 over the range of O_2 concentrations employed.

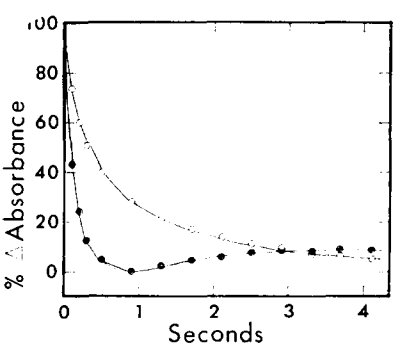

Fig. 4. Reaction of reduced *Pseudomonas* cytochrome oxidase with O_2 followed in the stopped-flow apparatus to monitor the slower absorbance changes at 460 nm (●) and at 549 nm (○). Conditions were the same as described in Fig. 2.

Similar rates are calculated from observations at 660 and 645 nm, the α-region of the heme d_1 component, although the absolute changes at these wavelengths are much less due to the smaller $\Delta\varepsilon$.

The oxidation of the heme *c* moiety of the enzyme, as indicated by the absorbance change at 549 nm, is essentially first order over most of the time range in Fig. 2. There is a slight deviation from first order within the first 50 ms or so of the reaction, but this appears to be due to a small contribution of heme d_1 to the absorbance in this region of the spectrum. When the enzyme reacts with 142 μM O_2 the first-order rate constant for the oxidation of heme *c* is about 4.5 s^{-1} and if the data are extrapolated to an infinite concentration of O_2, a limit rate constant of about 8 s^{-1} is reached.

The absorbance changes that are referred to above are those occurring within the first 500 ms after mixing the oxidase with relatively high concentrations of O_2. In addition, there are further and more complicated changes that occur between 500 ms and several seconds after mixing. These complex changes are dependent on the wavelength of observation and seem to be associated with the reaction of heme d_1. Fig. 4 shows the absorbance changes that occur at 460 and 549 nm between the time of mixing the reduced enzyme with O_2 and 4.1 s later. Although the reaction of the heme *c* at 549 nm appears to be straightforward and about 95 % complete at 4.1 s, the absorbance change at 460 nm changes direction after reaching a minimal value at about 900 ms. This reverse change is quite slow and takes about 3 s to reach a steady-state level which is maintained out to 4.1 s, the final point in these stopped-flow experiments.

These slow changes were monitored also in a conventional spectrophotometer where the oxidase solution was placed in an anaerobic cuvette fitted with a rubber serum cap. With the system under N_2 the oxidase was reduced by adding an anaerobic solution of 0.10 M potassium ascorbate (pH 7.0) by means of a gas-tight microsyringe inserted through the cap. The increase in absorbance of the oxidase band at 549 nm was used to monitor reduction; the addition of ascorbate was terminated when the enzyme was about 90 % reduced. Oxidation was initiated by adding from a gas-tight syringe a volume of air-equilibrated buffer equal to the volume of enzyme in the cuvette and quickly tilting the cuvette several times to mix the solutions. By the time observation in the spectrophotometer was begun 2–3 s had elapsed and the change at 460 nm was in the steady-state phase. This phase continued for about 7–8 s and was followed for about 5 s by a slow decrease in absorbance until no further change occurred. When the same experiment was performed with observation at 549 nm only a slow decrease in absorbance was seen and the change was complete about 10–15 s after mixing. It appears, therefore, that the heme d_1 undergoes some reduction during the latter stages of the reaction at a rate that is faster than its oxidation by O_2. However, this accounts for only a small proportion (< 10 %) of the total absorbance change at that wavelength.

Inhibition by cyanide

When 0.1 mM KCN was added to the reduced *Pseudomonas* cytochrome oxidase and this solution was mixed with air-equilibrated buffer in the stopped-flow apparatus, no changes in absorbance were observed. This result indicates that neither of the heme components of the oxidase undergoes a change in redox state when O_2

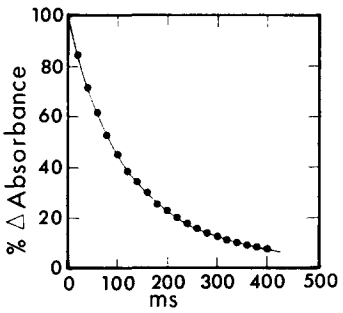

Fig. 5. Reaction of reduced *Pseudomonas* cytochrome oxidase with CO followed in the stopped-flow apparatus at 460 nm. The concentration of enzyme was 12 μM (expressed as heme *c*) and that of CO, 465 μM, after mixing. Other conditions were the same as described in Fig. 2.

is added to the reduced enzyme in the presence of cyanide. Any such change would be observed since the spectral forms of the free ferrous and ferric enzyme and of the liganded ferrous and ferric state differ significantly from one another.

Reaction with CO

Reduced *Pseudomonas* cytochrome oxidase reacts with CO to form a CO · enzyme complex. The changes in the absorption spectrum of this complex indicate that the CO reacts with the heme d_1 moiety of the oxidase, but not with the heme *c* component.

When 12 μM *Pseudomonas* cytochrome oxidase in its ascorbate-reduced form was reacted with 461 μM CO in the stopped-flow apparatus a rapid decrease in absorbance was observed at 460 nm. This change, illustrated in Fig. 5, has a half-time of about 85 ms. The second-order rate constant, k_{on}, calculated from these data is $1.77 \cdot 10^4\ M^{-1} \cdot s^{-1}$ with respect to CO. If this reaction is monitored over a period of several seconds a biphasic reaction becomes apparent in which the second phase is significantly slower than the first and has a pseudo-first-order rate constant of about $1\ s^{-1}$. The slower phase accounts for less than 10 % of the total absorbance change at 460 nm.

Titration of the enzyme with CO

When dithionite-reduced *Pseudomonas* cytochrome oxidase is titrated anaerobically at 25 °C with a solution containing CO (saturated at 1 atm.) and the absorbance changes at 460 nm are monitored, the results shown in Fig. 6 are obtained. By taking into account the amount of CO remaining in physical solution these data can be used to calculate an equilibrium constant for the reaction

CO · *Pseudomonas* cytochrome oxidase $\rightleftharpoons$ CO + *Pseudomonas* cytochrome oxidase

where the dissociation constant, K_d, has a value of $2.3 \cdot 10^{-6}$ M.

The rate constant, k_{off}, for the dissociation of CO from the oxidase can be calculated using the dissociation constant, K_d, of $2.3 \cdot 10^{-6}$ M described above and k_{on} of $1.8 \cdot 10^4\ M^{-1} \cdot s^{-1}$ calculated from the stopped-flow experiments. From these data k_{off} can be given a value of $0.041\ s^{-1}$.

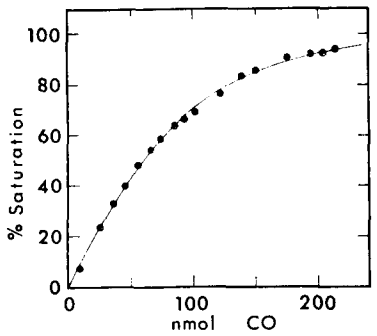

Fig. 6. Titration of reduced *Pseudomonas* cytochrome oxidase with CO. The ordinate gives the change in absorbance at 460 nm, expressed as a percentage of the total change, corresponding to the amount of CO shown in the abscissa. The concentration of enzyme was 37.3 μM (expressed as heme d_1) in 3.0 ml of 0.05 M potassium phosphate (pH 6.6). The titration was performed as described in Methods. Other conditions were: temperature, 25 °C; light path, 1 cm.

DISCUSSION

The stopped-flow experiments indicate that when reduced *Pseudomonas* cytochrome oxidase reacts with O_2 the heme d_1 moiety is oxidized at a faster rate than the heme *c* component. Since cyanide and CO each react with the heme d_1 group, but not with the heme *c* [6, 11] and each reagent blocks the reaction of the enzyme with O_2 [6], it seems likely that there is a sequential transfer of electrons through the enzyme to O_2:

heme $c \rightarrow$ heme $d_1 \rightarrow O_2$

Thus, *Pseudomonas* cytochrome oxidase reacts with O_2 in two steps; one is the reaction of heme d_1 with O_2 and the second, the reduction of heme d_1 by heme *c*. The first step is accurately second order and proceeds with a rate constant of $5.7 \cdot 10^4\ M^{-1} \cdot s^{-1}$ at 20 °C. The second step is significantly slower than the first and is first order with a limiting rate constant of about $8\ s^{-1}$. When an excess of an electron donor to the enzyme is present a larger proportion of heme *c* than of heme d_1 is maintained in the reduced form indicating further that the rate-limiting step is the reduction of heme d_1 by heme *c*. This conclusion is also supported by our earlier studies [10], which demonstrated that the reduction of heme *c* in the oxidase by electrons from the *Pseudomonas* copper protein is more rapid than the reduction of the heme d_1.

In addition to the more rapid rates of oxidation that occur initially after mixing the reduced enzyme with O_2, there are some slower changes that occur over a period of several seconds. These changes are small compared to the rapid changes in absorbance and seem to involve a condition where the rate of reduction of heme d_1 becomes slightly faster than the rate of its oxidation. The cause of these slower changes is not clear, but could be due to any of several factors, among which are (1) a small population of inhibited enzyme molecules whose heme d_1 is oxidized less rapidly than it is reduced by heme *c*, (2) the formation of an inhibitory enzyme-product complex where the product may be a partially reduced form of O_2, and (3) a slow reduction of the oxidized heme components by some residual ascorbate. Whatever the

cause of these slow changes they appear minor in magnitude compared to the more rapid changes and do not appear to affect the conclusions based on the more rapid absorbance changes.

Recent evidence indicates that *Pseudomonas* cytochrome oxidase consists of two equivalent subunits, each of which contains one heme *c* and one heme d_1 moiety [4]. The dimeric form of the purified enzyme therefore contains four potential reducing equivalents when the enzyme is fully reduced. These four equivalents could provide a source of electrons for the reduction of O_2 to water. The present results do not enable us to predict a mechanism for the reduction of O_2, but they do suggest a need for further experiments to search for intermediates in the reduction of O_2 as well as to determine whether a pair of heme d_1 components are jointly involved in the reaction.

The rates at which *Pseudomonas* cytochrome oxidase reacts with O_2 are much slower than those rates observed by Gibson and his associates [12] for the mitochondrial cytochrome *c* oxidase from beef heart. Thus, the second-order rate constant of $5.7 \cdot 10^4\ M^{-1} \cdot s^{-1}$ observed for the *Pseudomonas* enzyme is some three orders of magnitude lower than that obtained for the mitochondrial oxidase. Interestingly, there is much less difference in the rate constant for the reaction of the reduced oxidases with CO. Gibson et al. [12] calculated a rate constant of $7.2 \cdot 10^4\ M^{-1} \cdot s^{-1}$ for the reaction of the beef heart enzyme with CO while, in the present study, a rate constant, k_{on}, of $1.8 \cdot 10^4\ M^{-1} \cdot s^{-1}$ was calculated for the *Pseudomonas* enzyme. This value is practically identical to that of $2 \cdot 10^4\ M^{-1} \cdot s^{-1}$ reported recently by Parr et al. [14] for their preparation of *Pseudomonas* cytochrome oxidase. The rate constant, k_{off}, of $0.041\ s^{-1}$ for the dissociation of CO from the enzyme, which was calculated using the equilibrium constant, K_d, and the association rate constant, k_{on}, is not significantly different from the values of $0.03\ s^{-1}$ and $0.15\ s^{-1}$ found by Parr et al. [14] by displacing CO with NO or O_2.

In their recent paper Parr et al. [14] observed a clearly biphasic reaction when they mixed CO with their ascorbate-reduced oxidase. They pointed out that the complex reaction may result from heme-heme interaction and their data suggested the presence of positive co-operativity in the system. Earlier Parr et al. [15] presented evidence that precludes the use of dithionite as a reductant for *Pseudomonas* cytochrome oxidase in kinetic studies involving CO because it introduces anomalies into the reaction that are not observed when ascorbate is used to reduce the enzyme. The reaction of CO with our ascorbate-reduced oxidase also results in a biphasic reaction with the slower phase manifesting itself after about 400 ms. However, in our experiments the magnitude of the slower phase appears to be somewhat less than that observed by Parr et al. [15], although we have not explored the reaction with CO in the detail which they did. In any event, it is clear that the reactions of *Pseudomonas* cytochrome oxidase with CO and with O_2 deserve further investigation and the use of this enzyme as a model for mammalian cytochrome oxidase may prove to be more fruitful than even we first anticipated.

ACKNOWLEDGEMENTS

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