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EVIDENCE FOR WATER AS THE PRODUCT FOR OXYGEN REDUCTION BY CYTOCHROME CD

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Summary: Evidence is presented that water is the final product of electron donation to molecular oxygen by cytochrome cd from Paracoccus denitrificans when ferrocytochrome c acts as donor to cd. Negative evidence for the accumulation of superoxide and peroxide was obtained by rate effect experiments in the presence of superoxide dismutase, catalase, and peroxidase. Positive evidence for water was obtained by showing a 4 to 1 stoichiometric balance for rates of electron acceptance from ferrocytochrome c to rates of donation to molecular oxygen.

Paracoccus denitrificans contains a soluble, cd-type cytochrome which functions as the physiological nitrite reductase (1-3). A homologous enzyme isolated from Pseudomonas aeruginosa has been extensively studied (4-7). Enzymes of this class contain two c-type hemes and two d-type hemes distributed among two subunits of molecular weight about 60,000 daltons (8). The enzyme reduces NO_2^- to NO, with reducing equivalents provided by either cytochromes c or artificial donors (3-7). The enzyme also catalyzes the reduction of molecular oxygen. Although this latter reaction is now believed to be of no physiological importance (1), it is significant with regard to mechanistic understanding of the enzyme. The reactions with oxygen suggest the participation of intramolecular heme-heme electron transport events.

No data have been reported for the Paracoccus system on the product of reduction of O_2 by cytochrome cd. Little evidence is available for the analogous Pseudomonas system. One report implicated hydrogen peroxide as the product for reactions catalyzed by Pseudomonas cytochrome cd in which artificial donors such as ascorbate were employed (9). The experimental evidence consisted of observations of rate stimulation when catalase was added

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to reaction mixtures, but only when the addition was before the initiation of cd reaction. There exists some ambiguity about these experiments. A later paper on Pseudomonas cd failed to observe catalase activation (10). The original work could not account for different degrees of activation dependent on the particular donor used; it also did not account for catalase inhibition by ascorbate (11), one of the prime donors studied.

The purpose of this note is to present evidence obtained with Paracoccus cytochrome cd that water is the reduction product of O_2. Four possible products need to be considered, corresponding to the number of electrons transferred by the enzyme to O_2:

$O_2 + 1e^- \longrightarrow O_2^-$	superoxide	[1]
$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$	peroxide	[2]
$O_2 + 3e^- + 3H^+ \longrightarrow OH\cdot + H_2O$	hydroxyl radical	[3]
$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$	water	[4]

The hydroxyl radical has been dismissed as an unreasonable possibility. Its production would not be in accord with the known symmetry of metal redox centers in the enzyme. Three electron events are unprecedented in oxidation/reduction enzymes. This then leaves superoxide, peroxide, and water as realistic possibilities.

Materials and Methods

Paracoccus denitrificans cytochrome cd was prepared according to the method of Newton (2) or by a modified procedure to be described elsewhere. Because of low cellular content (2) and low yields in the purification some of the experiments to be described were performed with semipurified enzyme where the ratio of absorbance at 280 to absorbance at 409 was 6. However, key results were checked with purified material where A_{280}/A_{409} was less than or equal to 1. It should be pointed out that the semipurified material has been freed from other cytochromes. Its visible spectrum contains only bands characteristic of cytochromes cd (2,6). It focuses as a single green band on isoelectric focusing gels prepared according to Finlayson and Chrambach (12) at the expected pH of 4 (2). Horse cytochrome c was purchased from Sigma, rechromatographed on Sephadex G-75, then exhaustively dialyzed versus 20 mM tris base plus 10 μM EDTA before storage in 50 mM tris-cacodylate, pH 7.2. Horse cytochrome c is an efficient donor to Paracoccus cytochrome cd (Robinson and Timkovich, in preparation), although it is not to Pseudomonas cytochrome cd (13). Bovine catalase, horseradish peroxidase, and bovine superoxide dismutase were purchased from Calbiochem. Assays and activity units have been previously defined (14-16). All other reagents were commercial, analytical grade chemicals. Spectrophotometric measurements were performed on either a

Gilford Model 252 or a Cary 15 spectrophotometer. Oxygen polarographic assays were performed with a Yellow Springs Model 53 Clark oxygen electrode. All assays were done at 25°C. Cytochrome c was reduced with solid dithionite then chromatographed on short columns of Sephadex G-25 to remove excess reagent and by products. Changes in cytochrome c concentration were monitored at 550 nm using a difference extinction coefficient of 18.5 mM^{-1}. Paracoccus cytochrome cd was quantitated by means of its absorbance at 409 nm using an extinction coefficient of 150 mM^{-1}.

Results and Discussion

If superoxide were a product or a free intermediate during the aerobic oxidation of ferrocytochrome c by cd, it would be expected to decrease the net rate of disappearance of ferrocytochrome, because of the rapid reduction of ferricytochrome by superoxide in aqueous solution (16). A net disappearance of ferrocytochrome could still occur due to competition between ferrocytochrome oxidation catalyzed by cd, reduction back to the ferrous state by O_2^-, and the disproportionation reaction of O_2^-:

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \qquad [5]$$

Reaction 5 is catalyzed by superoxide dismutase (16). If superoxide were present during aerobic assays of cd, added superoxide dismutase would be expected to increase the rate of ferrocytochrome c disappearance by means of catalysis of disproportionation 5. Figure 1a demonstrates a typical experiment on the effect of superoxide dismutase. No rate changes are observed when superoxide dismutase is added in pulses as indicated or included initially in the assay.

If hydrogen peroxide were a product, it should be detectable through an effect by catalase on assays in which the rate of disappearance of O_2 was monitored. Catalase catalyzes the reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad [6]$$

Catalase added to an assay mixture which has been allowed to accumulate hypothetical peroxide as product should produce a jump in monitored O_2 as a result of reaction 6, or a rate change due to competition between reactions 2 and 6. Figure 1b demonstrates the effect of added catalase on an oxygen electrode

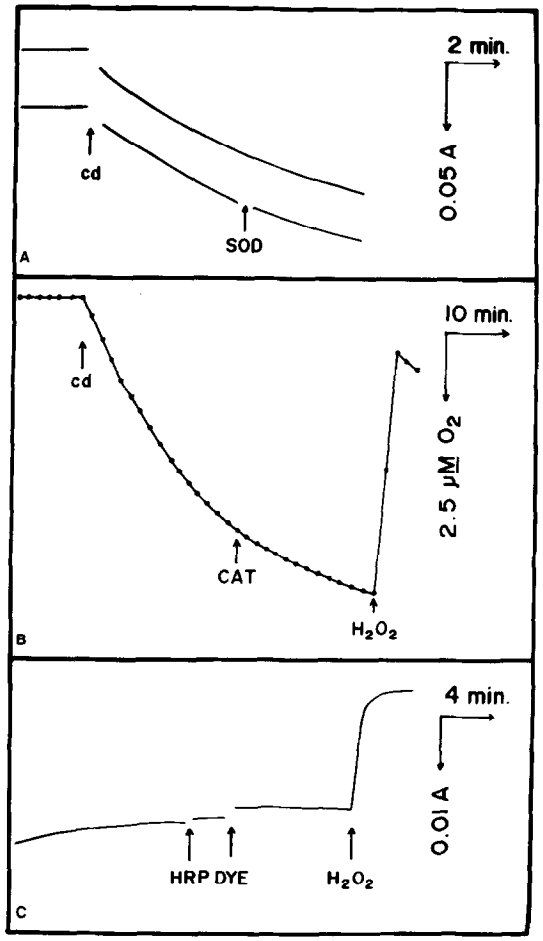

Fig. 1 A. Trace of a spectrophotometric assay at 550 nm for the oxidation of horse cytochrome c by Paracoccus cytochrome cd in which oxygen is the terminal electron acceptor. Top curve: 11.4 μM ferrocytochrome c in air saturated 10 mM phosphate buffer containing 0.25 mM EDTA at pH 6.0 in a total volume of 3.0 ml. Cytochrome cd was added at the first arrow to a final concentration of 0.082 μM. Bottom curve: same conditions, but 10 units of superoxide dismutase added at the second arrow. B. Plot of oxygen uptake for a polarographic assay of Paracoccus cd utilizing a Clark oxygen electrode. Data have been replotted from a recorder trace to obtain a convenient scale and have been corrected for a slight initial auto-oxidation of ferrocytochrome. Total volume was 3.0 ml containing 31.4 μM ferrocytochrome c and 0.124 μM cd added at the first arrow. Catalase, 900 units in 10 μl, was injected with a syringe at the second arrow and H_2O_2 at the third arrow to a final concentration of 9 μM. The peroxide injection is a control to demonstrate the activity of catalase in the reaction mixture with a known amount of substrate. C. Trace of a spectrophotometric assay at 460 nm for the oxidation of horse c by Paracoccus cd. Ferrocytochrome c, 12.0 μM, was oxidized by 0.145 μM cd in air saturated phosphate buffer, 50 mM, pH 6.0, and a total volume of 3.0 ml. The trace shows the end of the assay, after the reaction is more than 90% complete. The initial rise in absorbance at 460 nm is due to the spectral change accompanying the ferro to ferricytochrome conver-

monitored reaction. No effects were seen on reactions in which the cytochrome c donor concentration was varied between 10 and 300 μM and the cd concentration varied between 0.025 and 1.7 μM.

Hydrogen peroxide may also be detected by means of the horseradish peroxidase catalyzed oxidation of the dye o-dianisidine·2HCl, monitored at 460 nm (15):

$$\text{dye (reduced)} + H_2O_2 \xrightarrow{\text{HRP}} \text{dye (oxidized)} + 2H_2O \qquad [7]$$

Figure 1c shows negative results for detecting peroxide by this technique.

The preparations of cytochrome cd employed were assayed for inherent levels of catalase-like or peroxidase-like activity. Catalase activity was assayed in the oxygen electrode in the absence of an electron donor. Peroxidase activity was measured anaerobically in Thumberg type cuvettes in which the main well contained 10-20 μM ferrocytochrome c and 0.05 to 0.1 μM cd in 50 mM phosphate buffer pH 6, and the side arm 10 to 500 μM aqueous H_2O_2. Control experiments were necessary to correct for a slow rate of oxidation of ferrocytochrome c by peroxide. Expressed as turnover numbers, the inherent catalase activity of our preparation of cytochrome cd was less than 0.2 μM H_2O_2 per second per μM cd, and the inherent peroxidase activity less than 6×10^{-4} μM H_2O_2 per second per μM cd. These rates are essentially zero and represent upper estimates based on instrumental accuracy and our given supply of enzyme. They are approximately 10^6-fold slower than respective turnover numbers for bovine catalase and horseradish peroxidase (14-15).

Data in favor of water as the reduction product was obtained by comparing initial rates of reaction for spectrophotometric assays, which monitor the disappearance of ferrocytochrome c, with rates for polarographic assays, which monitor disappearance of O_2. If the net reaction catalyzed by Paracoccus cd is:

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sion. One unit of horseradish peroxidase was added at the first arrow. The peroxidase specific electron donor O-dianisidine was added at the second arrow to a final concentration of 0.1 mM. The addition of 5 μM H_2O_2 at the third arrow demonstrates the peroxidase activity in the presence of the additional mixture components.

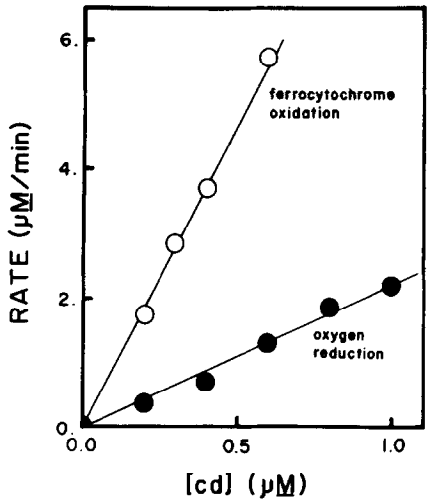

Fig. 2 Comparison of spectrophotometric and polarographic assays for Paracoccus cytochrome cd. In every case assays were performed in a total volume of 3.0 ml of air saturated 50 mM phosphate buffer, pH 6.0. For the spectrophotometric assays, the ferrocytochrome c concentration was 30 μM and the activity was measured as initial rates. For the polarographic assay, electron donation was provided by 30 μM ferrocytochrome c and 50 mM hydroquinone. Hydroquinone is an active electron donor to cytochrome c, maintaining a steady state in which the cytochrome c is 87% reduced during the assay. It is a poor donor to cytochrome cd and also shows very slow autooxidation rates with O_2. It serves to maintain a constant concentration of ferrocytochrome c for sufficient time that a reliable O_2 uptake trace may be measured. This technique is analogous to polarographic assays of mitochondrial oxidase in which ascorbate is used to maintain steady state levels of donor ferrocytochromes (17). Ascorbate cannot be used in the present case because of its high rate of direct reaction with cd. The rates expressed represent the stimulation of O_2 uptake caused by addition of cytochrome c to mixtures containing hydroquinone plus enzyme. The ratio of the slopes of the two straight lines represents the ratio of initial rates for ferrocytochrome c oxidation and O_2 reduction and this is computed to be 4.1.

$$4 \text{ ferrocytochrome } c + O_2 + 4H^+ \xrightarrow{cd} 4 \text{ ferricytochrome } c + 2H_2O \quad [8]$$

then the rate of ferrocytochrome disappearance should be four times the rate of O_2 disappearance. Figure 2 compares the observed rates for the reaction of cd in spectrophotometric and polarographic assays under equivalent conditions, as a function of enzyme concentration. The ratio of the slopes for the two lines is 4.1, in agreement with reaction 8.

By the elimination of other alternatives and the rate comparison above, water is concluded to be the ultimate product for electron donation to O_2 by Paracoccus cytochrome cd. Reaction 8 reflects the overall stoichiometry

of the enzyme catalyzed reaction. Data presented here do not provide information on possible enzyme bound or transient species, such as superoxy or peroxy-complexes, which may be intermediates in the catalytic cycle. Although these experiments have not been performed on Pseudomonas cytochrome cd, the homology between the Paracoccus and Pseudomonas systems (2) would argue that water is also a product in the latter case.

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