Turnover of Cytochrome c Oxidase from Paracoccus denitrificans*

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The heme aa₃ type cytochrome oxidase from Paracoccus denitrificans incorporated into vesicles with phospholipid reacts during turnover much as the oxidase from mitochondria does. The spectrophotometric changes observed at various wavelengths are closely similar, and the rate is about one-half of that for beef heart oxidase under the same conditions. The rate of appearance of oxidized cytochrome c on initiation of the reaction is also similar and depends on the previous treatment of the oxidase as described by Antonini, E., Brunori, M., Colosimo, A., Greenwood, C. and Wilson, M. T. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3128–3132. In terms of their model the resting Paracoccus enzyme is converted to the pulsed form during turnover. The effect is observed with both cytochrome c and heximine ruthenium as reductants. With the latter a 60-fold increase in rate is observed.

Initially, interest in bacterial heme aa₃ type cytochrome c oxidases (EC 1.9.3.1), reviewed in Ref. 1, was stimulated by the endosymbiosis hypothesis (2, 3) which suggests a possible evolutionary link to the mitochondria of eukaryotes. The oxidase from Paracoccus denitrificans has been examined in some detail (4–8) and is remarkably similar to the mitochondrial enzyme in some respects. The similarities include spectroscopic properties (4), interaction with cytochrome c (5, 6), reaction with oxygen and carbon monoxide (7), and proton pumping (8). These similarities are the more surprising because the two enzymes are structurally widely different, the Paracoccus enzyme having only two subunits rather than the seven or more of the mitochondrial enzyme, although immunological cross-reactions between Paracoccus and mitochondrial oxidase subunit II have been reported (1).

In this paper we report the results of experiments on the behavior of the Paracoccus enzyme during turnover. Except that the Paracoccus enzyme appears more dependent on phospholipid than the mitochondrial enzyme, the new results show that in turnover the two-subunit bacterial enzyme behaves almost precisely like the seven-subunit eukaryote one.

MATERIALS AND METHODS

Cytochrome c oxidase from Paracoccus denitrificans (ATCC 13543) was isolated as previously described (4) with a final purification step using a cytochrome c affinity column (9). Beef heart oxidase was purified as described in Ref. 10, and was the kind gift of Dr. D. C. Wharton, Department of Biochemistry, Northeastern University. The enzymes were incorporated into phospholipid vesicles (11), using asolectin partially purified by the method of Kagawa and Hacker (12) A preparation of this kind is referred to as reconstituted enzyme. Enzyme activity was measured at room temperature (24 ± 1 °C) with a Clark-type oxygen electrode with a Teflon membrane (Yellow Springs Instruments, Yellow Springs, OH) in a medium containing 0.1 M KF, pH 7.0, 30 mM horse heart cytochrome c (Sigma type VI), 10 mM potassium ascorbate, and 100 μM N,N,N′,N′-tetramethyl-p-phenylenediamine. Reconstituted Paracoccus cytochrome c oxidase assayed under these conditions had an average activity of 85 mol c/mol of aa₃/s. The comparable figure for mitochondrial enzyme was 135.

Protein was measured by the method of Lowry et al. (13) in 0.5% sodium dodecyl sulfide using bovine serum albumin as an internal standard. Oxidase heme was calculated from the peak of the reduced-oxidized difference spectrum at 605 nm using the published coefficients (4, 28).

Stopped flow kinetic experiments were performed with the apparatus of Gibson and Milnes (14) using either cytochrome c with ascorbate and tetramethyl-p-phenylenediamine, or heximine ruthenium (Alfa Products, Danvers, MA) with 5 μM ascorbate, as reductants. Oxygen concentrations were calculated from the data of Lange (15).

RESULTS

Reduction of Paracoccus Enzyme by Heximine Ruthenium—The reaction of Paracoccus oxidase with heximine ruthenium (II) in the absence of oxygen is biphasic with half-times of 0.14 and 27 s for the two phases using 50 μM heximine ruthenium at 11 °C with reconstituted enzyme. The corresponding half-times for the beef heart enzyme were 0.16 and 38 s, which compare reasonably with the values reported by Scott and Gray (16). The areas under the two distinct spectra obtained for the two phases by repeating the experiment with light of different wavelengths are of the same order. It is reasonable to suggest that the components are present in similar amounts. In the mitochondrial oxidase the slower component has been attributed to heme α₁ by the use of cyanide (16), an assignment supported by comparison with the redox difference spectra given by Gibson and Greenwood (17). The difference spectra for the two components of Paracoccus in stopped flow experiments are shown in Fig. 1, and closely resemble those for the mitochondrial oxidase (16, 30). Although the half-time for the reduction of heme α₁ is significantly shorter for the Paracoccus enzyme, the rates refer to an unnatural substrate, and in addition, the experiments were performed with resting enzyme, in the terminology of Brunori et al. (18).

Reaction with Cytochrome c—It has already been reported that purified Paracoccus oxidase requires added phospholipid if it is to be active in the usual assay system (4). It seems that the lipid is required to permit the interaction of oxidized oxidase with cytochrome c, since in experiments in which oxidase in 0.25% Tween 80 was mixed with 8 to 40 μM ferro cytochrome c there was no significant reduction of heme α over a period of some seconds. This result is sufficient to explain the inactivity of the purified enzyme in turnover experiments. Although this behavior is different from that of...
the usual preparations of the mitochondrial enzyme, the difference may be due more to the procedures used in making the preparations than to differences in the enzymes themselves, and it has been reported recently that beef heart mitochondrial enzyme requires 1 to 3 molecules of cardiolipin (diphosphatidyl glycerol) for activity (19).

When phospholipid is added to the Paracoccus enzyme the rate of reaction with cytochrome c is greatly accelerated. Specific anaerobic experiments directed to this point were not performed, but the rate of appearance of oxidized cytochrome c at the beginning of turnover was observed on many occasions. Under the conditions of Fig. 2A the concentration of oxidized cytochrome c reached one-half of the steady state value in 50 ms, and there was no clear difference between the resting and pulsed enzymes. This is between 2 and 3 times faster than for the beef heart enzyme which required 130 ms, for resting and 100 ms for pulsed enzyme, under the same conditions. Further interpretation of these data would require adoption of a specific model which can scarcely be justified at present.

Resting and Pulsed Enzyme—Over the last few years, evidence has been presented that oxidized cytochrome oxidase can populate two forms, one of which, called pulsed enzyme, shows faster intramolecular electron transfer than the other form, the resting enzyme (18, 20, 21). Experimentally, if a turnover reaction is initiated starting with reduced enzyme, it is in the pulsed state, whereas if the enzyme is oxidized, unless specific steps are taken, it is in the resting state. The rate and the progress curve of the reaction is different in the two cases, and it has been suggested that the pulsing phenomenon may be important in regulation of oxidase activity. An examination of the behavior of the Paracoccus enzyme in turnover was therefore made, since the function of the extra subunits of the mitochondrial enzyme might be related to the resting to pulsed transition.

The time course of the absorbance change for the Paracoccus and mitochondrial enzymes during a turnover reaction as followed at 550 nm (cytochrome c) and at 445 nm (heme a, when corrected for the contribution of cytochrome c) is presented in Figs. 2 and 3. Both enzymes show a clear pulsing effect with a higher rate of turnover reflected in a shorter time of reaction, and higher steady state concentrations of oxidized cytochromes c and a when the reaction is started with the enzyme in the reduced state. There are, however, significant quantitative differences between the enzymes. First, the turnover rate is about 2-fold higher for the pulsed mitochondrial enzyme than for pulsed Paracoccus enzyme whether this is estimated from the time required to exhaust oxygen or by integrating the area under the oxidized c progress curve. The extent of the observed pulsing phenomenon, measured by the difference in rates for resting and pulsed enzyme, is 2-fold for the mitochondrial enzyme as against the 20% increase for Paracoccus. When the turnover is started from the resting state, both enzymes show a transition from the resting to the pulsed state. This is most clearly seen in the mitochondrial enzyme as a progressive increase in the rate of turnover and hence of the concentration of oxidized c and oxidized a in the system. In neither case does the maximum rate for resting enzyme reach that observed with pulsed enzyme. A characteristic feature noted in experiments with the resting enzyme is also missing, namely, a sharp increase in rate just before

**Fig. 1. Kinetic spectrum for the reduction of Paracoccus enzyme.** In a stopped flow experiment the concentrations after mixing were 2.1 μM heme α reconstituted with 15 μg of asolectin/mg of protein, 5 mM ascorbate, and 50 μM ruthenium hexamine. The reaction was followed on two time scales for 0.6 and 80 s and a baseline taken after 2 min. The temperature was 11.3 °C light path 2 cm.

**Fig. 2. Turnover of resting and pulsed Paracoccus enzyme.** Stopped flow experiment. Concentrations after mixing: 2.4 μM heme α ( ), resting) and 2.3 μM α ( ), pulsed). Enzyme reconstituted with 20 mg of asolectin/mg of protein, 5 mM ascorbate, 0.25 mM N,N,N',N'-tetramethyl-p-phenylenediamine, 20 μM cytochrome c, 128 μM O₂, light path 2 cm, temperature 20.5 °C. A, reaction followed at 550 nm (cytochrome c); B, reaction followed at 445 nm (with correction for cytochrome c gives contribution of heme α).

**Fig. 3. Conditions as for Fig. 2, but beef heart oxidase, 2.7 μM, 25 mM ascorbate.**
oxygen is exhausted. It has been attributed to an increased probability of making the resting-pulsed transition at low oxygen concentration (20). The experiments reported here have been performed using reconstituted rather than detergent-stabilized enzyme, and this may influence the pulsing phenomenon. It may also be noted that both enzymes show an overshoot in approaching the steady state when in the resting form. This is because a larger population of oxidized enzyme is available to react with reduced cytochrome c during the first turnover than at any subsequent time.

Development of the Pulsing Phenomenon.—The transition of the resting enzyme toward the pulsed state during turnover can be visualized from the course of the absorbance at 550 nm. In Fig. 3A the rate of turnover increases from 35 to 50% of the rate reached by the pulsed enzyme during the course of the reaction, and the rate continues to increase until the oxygen is exhausted. With the Paracoccus enzyme the rate achieved by the resting enzyme quickly (during the first quarter of the reaction) reaches some 80% of the rate of the pulsed enzyme and shows little further change until the oxygen is exhausted. The implication is that, under the conditions of the experiment, not all of the enzyme is readily pulsed.

The progress of the pulsing phenomenon has also been followed by an entirely independent method using hexamine ruthenium as the reducing agent. As described under “Reduction of Paracoccus Enzyme by Hexamine Ruthenium,” reduction of heme a, is much slower than the reduction of heme a when this reagent is used with resting enzyme under anaerobic conditions. If, however, hexamine ruthenium is used as reductant in turnover, the rate of reduction of heme a at the end of the reaction is increased as much as 60- to 70-fold. If this experiment is repeated using several different concentrations of oxygen so that the enzyme has executed different numbers of turnovers before its final reaction with excess reductant, the proportion of slow reaction changes progressively with change in the concentration of oxygen at the beginning of turnover, giving a measure of the amount of enzyme which remains in the resting form at the time when oxygen has been exhausted. Data collected for the Paracoccus enzyme and for the beef heart enzyme in a parallel experiment are shown in Fig. 4. The mitochondrial enzyme shows a much larger change in the range of observation than Paracoccus suggesting that Paracoccus needs many fewer turnovers for pulsing to occur than the mitochondrial enzyme does. In terms of the model proposed by Wilson et al. (20) this would require that the rate of change of the newly reduced enzyme from the resting to the pulsed form take place rapidly enough to compete successfully with the very rapid oxygen reaction, and so be of the order of 10³ s⁻¹ as compared with the estimate of 50 s⁻¹ given by (20) for the mitochondrial enzyme stabilized with Tween 80. This is by far the largest difference, numerically, between the two enzymes which has so far been found.

The actual change in rate of reduction of heme a, for the two enzymes is given in Table I, and is much larger than the change in rate of reaction with cytochrome c found by Wilson et al. (20).

DISCUSSION

The experiments reported here show that the Paracoccus and mitochondrial enzymes are similar in the series of reductive reactions involved in turnover with both cytochrome c and hexamine ruthenium as reducing agents. Further, the Paracoccus enzyme shows pulsing behavior as already described for the mitochondrial enzyme with cytochrome c (18, 20, 21). An analogous pulsing effect occurs with hexamine ruthenium, and it too is common to both enzymes. Qualitatively at least, no difference has yet been found between the oxidases which might reasonably be used to assign a function to the extra subunits of the mitochondrial oxidase. There are quantitative differences but these are usually quite small. For example, the turnover rates recorded under the same conditions differed by a factor of 1.5 in favor of the mitochondrial enzyme, and a margin of this size need not persist over a wide range of experimental conditions.

There is a similar difference in proton pumping activity which is 0.6H⁺/electron for Paracoccus and 1.1H⁺/electron for the mitochondrial enzyme (8). A body of evidence reviewed in Ref. 23 suggests that mitochondrial subunit III is involved in proton translocation, but presumably in a regulatory rather than in an obligatory capacity. A regulatory function for the smaller mitochondrial subunits has been suggested by Kadenbach and Merle (24) and is, of course, compatible with our results. Our failure to find clear evidence of such a function does not mean that it does not exist, since it may well reflect the use of unsuitable experimental conditions. The case of 2,3-diphosphoglycerate and hemoglobin whose functional effect was not discovered until 1968 offers a possible analogy. There is also a recent report (29) that the smaller units of bovine cytochrome c oxidase from different tissues are dissimilar.

The use of hexamine ruthenium as a reducing agent has allowed the ready separation of the difference spectra attributable to valence change of heme a and a. These are quite similar in amplitude and form to those for the mitochondrial enzyme reported by Scott and Gray (10, 17). It should be emphasized that these spectra are significantly different from another pair of spectra which have been separated kinetically during the oxidation of Paracoccus by molecular oxygen (7). This second pair was originally assigned to the oxidation of hemes a and

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**Table I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half-times (sec)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracoccus</td>
<td>29</td>
<td>66</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>52</td>
<td>75</td>
</tr>
</tbody>
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**Fig. 4. Progress of pulsing phenomenon.** In a stopped flow experiment varying amounts of oxygen were mixed with Paracoccus enzyme (1.9 μM heme a after mixing) reconstituted with 10 mg of ascorbate/mg of protein or bovine enzyme (2.8 μM heme a, in 0.225% Tween 80). The solutions also contained 5 mM ascorbate and 50 μM hexamine ruthenium. Path 2 cm, 11 °C. The enzyme was assigned to resting and pulsed states by splitting the time course of final reduction of the enzyme into its slow and fast components with the rates given in Table I. The excursions analyzed was 85% of the whole (Paracoccus, ⚫) and 90% for bovine enzyme (⚪).
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a, by Greenwood and Gibson (25) working with the mitochondrial enzyme. It has recently been pointed out that this assignment cannot be correct (30) and that spectrophotometrically distinct species must be populated during the oxidative and reductive reactions. The observations reported here, taken together with the spectra reported in Ref. 7, show that both types of enzyme react by means of very similar intermediates, in agreement with the results of low temperature experiments (26, 27).

So far, attention has been concentrated on the similarities between the two oxidases. There are some differences which seem to be well outside the range of experimental error. The most striking is in the readiness with which the pulsing phenomenon occurs. As mentioned under “Results,” the rate of transfer of resting reduced enzyme to pulsed reduced enzyme must be of the order of 50 times faster in Paracoccus than in mitochondrial oxidase when using hexamine ruthenium as reductant. If pulsing is important in the regulation of oxidase activity, this difference between the enzymes might be highly significant since in the Paracoccus enzyme the resting state would scarcely be populated during turnover. The second large difference is in the reaction with cytochrome c in the absence of added phospholipid. Although experimentally striking to the point of being a qualitative rather than a quantitative difference, Vik et al. (19) have recently reported that 1 to 3 molecules of diphasphatidyl glycerol (cardiolipin)/oxidase molecule are required for optimal activity of beef heart oxidase, so the difference may mean no more than that lipid is more effectively removed in the preparation of the Paracoccus enzyme.

Finally, it has been shown that pulsing occurs with hexamine ruthenium as well as with cytochrome c, and in terms of relative rates is some 10 times more marked with ruthenium. The values of the absolute rates suggest that the two phenomena (with c and Ru) may not be particularly closely related. According to the model of Wilson et al. (20), pulsing reflects the speeding up of electron transport within the oxidase which should not, in principle, depend on the nature of the external donor. In fact, intraoxidase transfer to a, is between 1 and 2 orders of magnitude slower after reduction of a with hexamine ruthenium than after reduction by cytochrome c.

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REFERENCES

Turnover of cytochrome c oxidase from Paracoccus denitrificans.
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