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Reaction of the *Escherichia coli* quinol oxidase cytochrome bo_3 with dioxygen: The role of a bound ubiquinone molecule

(electron transfer/oxygen reduction/cytochrome oxidase/heme-copper oxidases)

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ABSTRACT **We have studied the kinetics of the oxygen reaction of the fully reduced quinol oxidase, cytochrome bo_3, using flow-flash and stopped flow techniques. This enzyme belongs to the heme-copper oxidase family but lacks the Cu_A center of the cytochrome *c* oxidases. Depending on the isolation procedure, the kinetics are found to be either nearly monophasic and very different from those of cytochrome *c* oxidase or multiphasic and quite similar to cytochrome *c* oxidase. The multiphasic kinetics in cytochrome *c* oxidase can largely be attributed to the presence of Cu_A as the donor of a fourth electron, which rereduces the originally oxidized low-spin heme and completes the reduction of O_2 to water. Monophasic kinetics would thus be expected, *a priori*, for cytochrome bo_3 since it lacks the Cu_A center, and in this case we show that the oxygen reaction is incomplete and ends with the ferryl intermediate. Multiphasic kinetics thus suggest the presence of an extra electron donor (analogous to Cu_A). We observe such kinetics exclusively with cytochrome bo_3 that contains a single equivalent of bound ubiquinone-8, whereas we find no bound ubiquinone in an enzyme exhibiting monophasic kinetics. Reconstitution with ubiquinone-8 converts the reaction kinetics from monophasic to multiphasic. We conclude that a single bound ubiquinone molecule in cytochrome bo_3 is capable of fast rereduction of heme *b* and that the reaction with O_2 is quite similar in quinol and cytochrome *c* oxidases.**

Cytochrome bo_3 of *Escherichia coli* is a member of the quinol oxidase subfamily of the respiratory heme-copper oxidases. The quinol oxidases share with the cytochrome *c* oxidases a common motif of three metal centers: a binuclear heme-copper center where the O_2 chemistry occurs and a low spin heme, which serves as the immediate electron donor to this oxygen reduction site. Cytochrome *c* oxidases contain an additional metal center, Cu_A, which is the point of electron entry from cytochrome *c* (1). Cu_A is not present in the quinol oxidases, apparently because they are adapted to a very different type of electron donor (2). In cytochrome bo_3, hemes B and O occupy the low spin and the binuclear site, respectively (when so bound they are denoted hemes *b* and o_3; see refs. 3 and 4). This is why its spectroscopic characteristics differ considerably from the aa_3-type cytochrome *c* oxidases, as well as from the aa_3-type quinol oxidase from *Bacillus subtilis*, even though the latter is more closely related in amino acid sequence to the bo_3 enzyme (5).

The kinetics of the reaction of heme-copper oxidases with O_2 has been studied most intensively with bovine heart cytochrome aa_3 and the so-called flow-flash technique (6), monitored by optical and time-resolved resonance Raman spectroscopy (for a review, see ref. 7). The reaction of the fully reduced enzyme, which can deliver four electrons to oxygen, is a multiphasic process (8). The initial binding of O_2 ($\approx$120,000 s^{-1}) is followed by essentially simultaneous oxidation of both hemes by electron transfer to the bound oxygen ($\approx$30,000 s^{-1}; see refs. 7 and 9–12). Subsequently, the low-spin heme is partially rereduced by electron redistribution from Cu_A ($\approx$10,000 s^{-1}; see ref. 1). When the reaction is monitored at the peak wavelength of the reduced hemes, this latter phase gives rise to a distinct plateau or even a slight reversal of direction in the time course of the reaction, because it represents heme rereduction in a process otherwise dominated by heme oxidation. Finally, in a step that completes the reduction of O_2 to water, the low-spin heme and Cu_A both become fully oxidized ($\approx$1000 s^{-1}).

Since the quinol oxidases contain only three of the four metal centers found in the cytochrome *c* oxidases, the kinetics of the reaction with oxygen may be expected to exhibit fewer phases. In the absence of a fourth electron from Cu_A, neither the rereduction of the low-spin heme nor the final step in the reduction of O_2 to water should take place. Instead, after the oxidation of the two hemes and Cu_B (the copper ion of the oxygen reduction site), the reaction should end, leaving a ferryl intermediate trapped at the oxygen reduction site. However, such a prediction assumes that the oxygen reaction is basically the same in the quinol and the cytochrome *c* oxidases.

Precisely this behavior has been found in the cytochrome aa_3-600 quinol oxidase from *B. subtilis*, which is a close relative of the *E. coli* cytochrome bo_3 although it has different hemes (5). Lauraeus *et al.* (13) found that the product of the reaction between the fully reduced aa_3-600 and O_2 has the characteristic spectrum of the ferryl intermediate. Hill (14) reported only one rapid phase of heme oxidation and concluded that the reaction must have terminated with the ferryl intermediate.

The study of this reaction with cytochrome bo_3 has proved less straightforward. Orii *et al.* (15, 16) have reported that depending on the procedure of enzyme isolation (the presence or absence of chloride), either monophasic or multiphasic kinetics could be observed. Most significantly, they concluded that the reaction of cytochrome bo_3 with oxygen is mechanistically very different from that of cytochrome *c* oxidase.

We now report, in agreement with Orii *et al.* (15, 16), that either monophasic or multiphasic kinetics can indeed be observed with cytochrome bo_3, depending on the conditions of enzyme isolation. We find, however, that the determining factor is the presence or absence of bound ubiquinone in the enzyme. Enzyme that contains this extra electron donor consistently exhibits multiphasic kinetics, very much like those seen with cytochrome *c* oxidase. When this quinone is absent, a fast monophasic reaction is observed, leading to a trapped ferryl intermediate. We conclude that the reaction of cytochrome bo_3 with O_2 is qualitatively similar to that of cyto-

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Abbreviations: DM, *n*-dodecyl β-D-maltoside; Q-8, ubiquinone-8; TX, Triton X-100.

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chrome *c* oxidase, the bound ubiquinone rereducing the low-spin heme much as Cu_A does in the related enzyme.

MATERIALS AND METHODS

Enzyme Samples. Cytochrome bo_3 was genetically modified with a C-terminal histidine "tag" in subunit II of the enzyme (bacterial strain GO 105 with the plasmid pJRHisA; Jon N. Rumbley and Robert B. Gennis, personal communication). This enabled isolation of homogeneous cytochrome bo_3 enzyme in one step, using a metal chelate affinity column and elution with an imidazole gradient.

Cells were grown aerobically in a fermenter (Medical Braun) containing 15 liters of 1% (vol/vol) lactate minimal medium and harvested in the midexponential phase of growth. Cells (40 g) were washed with 20 ml of 500 mM NaCl/10 mM Tris·HCl, pH 7.5 per g of cells. Lysozyme treatment was done in 20 ml of 200 mM Tris·HCl, pH 7.5/2.5 mM EDTA per g of cells. After an osmotic lysis step in 20 ml of 10 mM potassium phosphate/2 mM EDTA per g of cells, membranes were washed with 5 mM imidazole/300 mM NaCl/20 mM Tris·HCl, pH 7.8. Washed membranes were solubilized with 1% (wt/vol) *n*-dodecyl β-D-maltoside (DM) (Anatrace, Maumee, OH) or with 1% (wt/vol) Triton X-100 (TX) (Boehringer Mannheim) and 1% (wt/vol) *n*-octyl β-D-glucoside (Anatrace) in 5 mM imidazole/300 mM NaCl/20 mM Tris·HCl, pH 7.8/5–10 mg of protein per ml. After ultracentrifugation, the supernatant was applied to an Ni-NTA-agarose (Qiagen, Chatsworth, CA) column, which had been equilibrated with 0.05% DM, or 0.1% TX, in 5 mM imidazole/300 mM NaCl/20 mM Tris·HCl, pH 7.8. The column was washed with 3 bed vol of the equilibration buffer. The enzyme was eluted from the column with a 5–110 mM imidazole gradient (10 bed vol). Fractions containing pure four-subunit cytochrome bo_3 were pooled and concentrated using a stirred Amicon cell with a YM 100 membrane. To lower the concentrations of imidazole and NaCl in the preparations, samples were diluted 1:10 in 20 mM Tris·HCl (pH 7.8) with detergent and concentrated again.

Chloride-Free Samples. Chloride-free samples were prepared the same way, except that sulfate was substituted for chloride in all steps, including cell culturing. All experiments shown here were performed with enzyme isolated in the presence of chloride.

Enzyme Concentrations. Enzyme concentrations were determined from the pyridine hemochrome spectrum, using the molar absorptivity of heme B at the absorption peak position of the 1:1 combination of hemes B and O and from the dithionite reduced minus oxidized α band of the enzyme (2, 17).

Ubiquinone-8 Determination. Ubiquinone-8 (Q-8) was determined by two different procedures. Method 1: Extraction with ethanol/*n*-hexane, 2:5 (vol/vol), and determination by reverse-phase HPLC and UV detection, as described by Takada *et al.* (18), with 1 μg of ubiquinone-9 (Sigma) as internal standard. Method 2 (19): 3–4 nmol of enzyme was diluted to 0.5 ml with 40 mM Tris·HCl and thoroughly mixed with 2.5 ml of 60:40 methanol/light petroleum ether (bp, 40–60°C) in 10-ml glass tubes. The light petroleum layer was separated by centrifugation for 5 min at 1500 × *g*, and the residue was reextracted with 1 ml of light petroleum ether. Combined extracts were evaporated under N_2 and dissolved in 0.5 ml of ethanol/*n*-heptane (75:25). The amount of Q-8 was determined spectroscopically from oxidized and oxidized minus reduced spectra as described by Kröger (19).

Reconstitution of Ubiquinone-8. *Procedure I.* The detergent of TX enzyme was changed to 0.2% DM in a 2 ml of Ni-NTA-agarose column; 0.3 ml of this enzyme (40 μM) was incubated with 1 ml of *E. coli* RG145 membranes as a source of Q-8 (40 mg of protein per ml) for 1 h, after which 1% DM was added to solubilize the membranes. After 5 min of centrifugation, the supernatant was run through an Ni-NTA-agarose column. Cytochrome bo_3, which is without the histidine tag in the RG145 membranes, was eluted with 25 mM imidazole and the original histidine-tagged cytochrome bo_3 was eluted with 150 mM imidazole in buffer (0.2% DM/300 mM NaCl/20 mM Tris·HCl, pH 7.8). The latter enzyme was then concentrated with a Centricon 100 (Amicon) concentrator, diluted at 1:10 with 0.2% DM/20 mM Tris·HCl, pH 7.8, and concentrated again.

Procedure II. Q-8 was purified from *E. coli* RG145 membranes according to Kröger (19). The detergent of the histidine-tagged enzyme purified with TX was changed to 0.2% DM in a 2-ml Ni-NTA-agarose column; 0.5 μmol of Q-8 was added in 0.5% DM solution to one sample of enzyme (20 nmol), whereas no quinone was added to the other. Both samples (20 nmol of bo_3) were incubated overnight on ice, after which they were run through the Ni-NTA agarose column in 0.2% DM/5 mM imidazole/300 mM NaCl/20 mM Tris·HCl, pH 7.8. Enzyme was eluted with 150 mM imidazole in the buffer. The samples were then treated as described above before measurements.

Flow-Flash Measurements. The kinetics of the reaction of fully reduced cytochrome bo_3 with oxygen were followed on a microsecond to millisecond time scale by the flow-flash technique (6). The fully reduced, CO-inhibited enzyme was mixed with oxygen-saturated buffer in the dark (mixing ratio, 1:5; enzyme to oxygen buffer), and the reaction was then initiated by photolyzing the CO from the enzyme with a laser flash (532 nm; 180 mJ; Brilliant, Quantel, Les Vlis Cedex, France). The subsequent reaction was followed optically using a dual-beam kinetic spectrophotometer built in-house: As a probe source, the light from the xenon flash lamp (Zeiss BL40) was passed through a monochromator and then split into two beams by partial reflection from a glass coverslip. The transmitted beam was directed through the sample cuvette and into a photomultiplier tube. The reflected beam, which did not pass through the sample was directed into a second photomultiplier tube. In this way, the probe light intensity could be measured before and after passing through the sample. The signals from the two photomultiplier tubes were recorded by separate digitizers and used to calculate time-dependent absorbance changes in the sample (see also refs. 10 and 20).

Stopped Flow Measurements. Although our flow-flash instrument gives excellent time resolution, it is limited to measuring at one wavelength at a time. We were able to obtain spectra during the reactions, to millisecond resolution, using a stopped-flow spectrophotometer equipped with a diode array detector (Unisoku Instruments). The reaction of fully reduced cytochrome bo_3 with oxygen was carried out by mixing dithionite-reduced enzyme with oxygen-saturated buffer (no CO). The enzyme solution was placed in one of the input reservoirs and made anaerobic by flushing with water-saturated N_2. The gas jet was directed at the liquid surface so that the solution was mixed without producing detergent bubbles. After ≈20 min, a small volume of dithionite solution was added to achieve a dithionite concentration of ≈200 μM. The other input reservoir was filled with buffer through which O_2 gas was bubbled. Thus, after mixing, the oxygen concentration is ≈600 μM compared to a dithionite concentration of 100 μM. The cytochrome bo_3 should react more quickly with the oxygen than with the dithionite, but the large excess of oxygen would be expected to destroy the remaining dithionite, forestalling any rereduction of the enzyme. To prevent interference from by-products of the dithionite–oxygen reaction, catalase was added to the enzyme sample.

Data Analysis. The spectrum presented in Fig. 1*B* was obtained by a global kinetic fit to the time–wavelength–absorbance surface from the stopped-flow instrument. The algorithm assumes that kinetic behavior at all wavelengths can

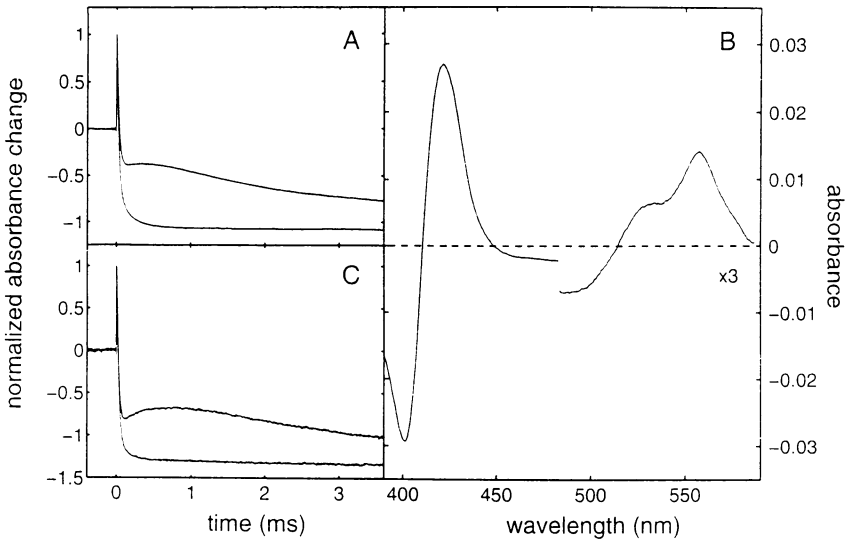

FIG. 1. Reaction of cytochrome bo_3 with oxygen. (*A*) Flow-flash experiments at 430 nm. Upper trace, enzyme prepared using only DM as a detergent (average of nine transients); lower trace, enzyme prepared using TX (average of 12 transients). The jump at time 0 is due to photolysis of the heme iron-CO bond by the laser flash (normalized to 1.0). Syringe A (1 ml), 20 μM cytochrome bo_3; (buffer, pH 7.0), 100 mM Mops, 0.1% DM, 60 μg of catalase per ml, 0.2 mM dithionite, 1.2 mM CO. Syringe B (5 ml), 1.2 mM oxygen, same buffer including catalase. All concentrations are before mixing. Mixing ratio, 1:5; total volume of each shot, 400 μl. (*B*) Product of the reaction of cytochrome bo_3 with oxygen by stopped-flow mixing. Spectrum was obtained by following the decay of the product of the fast reaction to the fully oxidized enzyme. The major component from a global kinetic fit is shown (time constant, 0.56 s^{-1}). Syringe A, 3 μM cytochrome bo_3 (prepared using Triton X-100), 100 mM Mops, 0.1% DM, 60 μg of catalase per ml, 0.2 mM dithionite. Syringe B, 1.25 mM oxygen, 100 mM Mops, pH 7.0 (no detergent). All concentrations are before mixing. Mixing ratio, 1:1. (*C*) Flow-flash experiments as in *A*. Lower trace, TX-isolated enzyme where DM was substituted for TX (control); upper trace, detergent substitution as above, but with added Q-8 (see procedure II in *Materials and Methods*).

be described in terms of a small number (two to four) of exponential components (26).

RESULTS

Fig. 1*A* shows the kinetics at 430 nm when the fully reduced cytochrome bo_3, prepared using either DM or TX reacts with O_2. The reduced enzyme, caged by CO, is mixed with O_2 in the dark. Then the reaction is initiated by a laser flash, which produces a fast initial absorption increase due to photolysis of the heme o_3-CO bond, after which the O_2 reaction ensues.

In enzyme isolated with DM (Fig. 1*A*, upper trace), the time course of the reaction appears much like it does in cytochrome *c* oxidase. Photolysis is followed by a fast oxidation phase, which ends abruptly at ≈120 μs. Subsequently, a slight rise in absorption leads to a plateau, after which a much slower decrease in absorption continues from 500 μs onward. In enzyme isolated using TX (Fig. 1*A*, lower trace), the process is dramatically different. Here, the first oxidation phase continues, apparently to completion; the plateau and slow phase are not observed at all. These results for enzyme prepared with TX and DM are similar to those described by Orii and co-workers (15, 16) for enzyme isolated in the presence and absence of chloride salts, respectively (see *Discussion*).

In cytochrome *c* oxidase, the plateau in the kinetic time course (sometimes there is a slight absorbance increase) has been attributed mainly to rereduction of the low-spin heme by Cu_A (see Introduction). Thus, the present results would be consistent with the idea that rereduction of the low-spin heme occurs in the DM enzyme but not in the TX preparation. Since cytochrome bo_3 lacks the Cu_A center, the presence of another endogenous electron donor might reasonably explain the kinetics of the DM enzyme. Q-8 is the native electron donor for this enzyme, and therefore a molecule of bound ubiquinone seems to be a likely candidate. Consistent with this, we find that enzyme prepared using DM retains ≈1 equivalent of Q-8, while enzyme prepared using TX contains none (Table 1).

If this explanation is correct, the fully reduced TX enzyme should be able to deliver only three electrons to bound O_2, and the reaction would thus be expected to lead to a trapped ferryl intermediate. Fig. 1*B* shows the spectrum of the final product of the fast reaction between fully reduced TX enzyme and O_2 as it relaxes to the fully oxidized enzyme over a period of several seconds. The α band at 557 nm and the Soret band at ≈420 nm are identical to those ascribed to a ferryl compound in cytochrome bo_3 (21).

Fig. 1*C* shows an experiment in which we attempted to reverse the effect of the TX treatment on the enzyme. As shown in the lower trace, returning the TX enzyme to DM did not change the monophasic kinetics, but by treating the enzyme with both DM and Q-8 (upper trace), the multiphasic kinetic behavior could be restored. Similar results were obtained with another reconstitution procedure in which a ubiquinone-free enzyme solution was allowed to equilibrate with native Q-8-containing bacterial membranes (see procedure I in *Materials and Methods*).

DISCUSSION

Although the time course of the redox processes in the flow-flash reaction of cytochrome *c* oxidase is relatively well understood, the corresponding reaction of the *E. coli* cytochrome bo_3 has been more problematic. Initially, multiple

Table 1. Q-8 content of cytochrome bo_3 preparations

Enzyme preparation	Q-8/bo_3
bo_3 TX	0.0
bo_3 TX	0.0
bo_3 TX	0.0*
bo_3 TX	0.2*
bo_3 DM	1.2
bo_3 DM	1.1

Q-8 was determined by method 2, except in samples marked with an asterisk, where method 1 was used (see *Materials and Methods*). Results of independent measurements with six different enzyme preparations are shown. TX, enzyme isolated with TX; DM, enzyme isolated with DM, both in Cl-containing medium.

phases of electron transfer were observed (22), but at least some of this could be ascribed to enzyme preparations that were heterogeneous in heme composition (10, 15). Working with homogeneous cytochrome bo_3, Orii and co-workers (15, 16) nevertheless observed both monophasic and multiphasic oxidation kinetics, depending on how the enzyme was isolated. They concluded that the fast monophasic reaction kinetics represented complete reduction of O_2 to water in a reaction kinetically controlled by O_2 binding to the heme, a mechanism fundamentally different from that of the cytochrome *c* oxidases. This interpretation was, at least in part, based on the finding by Sato-Watanabe *et al.* (23) that cytochrome bo_3 contains one tightly bound molecule of Q-8. With our isolation procedure, a single Q-8 molecule is retained when DM is used as the detergent, but there is no bound Q-8 when TX is used. We observed monophasic oxidation kinetics only in the latter case. Moreover, the end product of the monophasic reaction was shown to be the ferryl intermediate, as expected for an enzyme capable of donating only three electrons to bound O_2. Hence, the multiphasic kinetics can clearly be explained as due to the presence of a bound molecule of Q-8 that is capable of quickly rereducing heme *b*, by analogy with the role of Cu_A in the kinetics of cytochrome *c* oxidase (see Introduction). Our finding that the Q-8-free enzyme can be reconstituted with Q-8 to restore multiphasic kinetics strongly supports this interpretation.

Orii *et al.* (16) observed multiphasic kinetics in enzyme prepared in the absence of chloride salts. They proposed that Cl^- is needed to maintain an enzyme conformation in which fast heme-heme electron transfer can take place. However, Cl^- addition did not restore monophasic kinetics. We have observed that the inclusion of chloride in the isolation medium significantly decreases the amount of Q-8 retained in the enzyme preparations compared to experiments where sulfate was substituted for chloride (data not shown). It seems likely that under other conditions of enzyme isolation, depending on both detergent and the conditions of chromatography, chloride may facilitate removal of endogenous ubiquinone more effectively than in the DM preparation used here, where the isolation procedure requires only a single step on the Ni affinity column. Such an effect could be caused by competition of Cl^- with ubiquinol (QH^-) or ubisemiquinone (24) for binding to a cationic site in the enzyme (25).

Sato-Watanabe *et al.* (23) described a high-affinity Q-8 binding site in cytochrome bo_3, which they suggested may be involved in electron transfer from a low-affinity ubiquinone-binding site to the low-spin heme *b*. The data reported here support this proposal, showing that a single relatively tightly bound molecule of Q-8 is capable of fast electron transfer to the low-spin heme, much like the role of Cu_A in the cytochrome *c* oxidases.

CONCLUSION

We have presented evidence for a single bound molecule of Q-8 in cytochrome bo_3, which is capable of fast electron transfer to the low spin heme *b*. When this quinone is present, the quinol oxidase reacts with O_2 in qualitatively much the same multiphasic way as does cytochrome *c* oxidase. The precise rate constants may well differ, however, and the very fast binding of O_2 in cytochrome aa_3 (a 120,000 s^{-1} phase) has not yet been kinetically resolved in cytochrome bo_3. This may be because the rate constant of this phase, and that of electron transfer which follows, are sufficiently similar to make such distinction difficult. Another difference relative to cytochrome aa_3 kinetics is the prolonged slow oxidation phase in Q-8-containing cytochrome bo_3. This may be explained by the fact that ubiquinol carries two electrons (one more than Cu_A), so that there may be an extra electron available for reduction of the low-spin heme *b*. In the absence of the bound Q-8 molecule, reduced cytochrome bo_3 is capable of donating only three electrons to bound O_2, yielding the ferryl intermediate in an essentially monophasic reaction. We conclude that the reaction with O_2 is very similar in the quinol and cytochrome *c* oxidases.

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