

## Role of Quinones in the Branch of the *Escherichia coli* Respiratory Chain that Terminates in Cytochrome *o*

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The role of quinones in the cytochrome *o* branch of the *Escherichia coli* respiratory chain was investigated by using mutant strains lacking the cytochrome *d* terminal oxidase complex. The only cytochromes present were cytochrome *b*<sub>556</sub> and the cytochrome *o* complex, consisting of cytochrome *b*<sub>555</sub>-*b*<sub>562</sub>. Mutant strains missing ubiquinone, menaquinone, or both were constructed in the cytochrome *d*-minus (*cyd*) background. The steady-state levels of cytochrome *b* reduction were examined and compared in these strains to assess the effects of the quinone deficiencies. The data clearly show that a ubiquinone deficiency results in a lower level of cytochrome *b* reduction in the steady state. The data are consistent with a simple model in which ubiquinone is placed on the dehydrogenase side of all the cytochromes in this branch of the respiratory chain. There is no evidence from these experiments for a role of quinones in the respiratory chain at any site besides this one.

The aerobic respiratory chain of *Escherichia coli* has been characterized by spectroscopic (19, 22-24), potentiometric (5, 7, 8, 14a, 20, 21, 25), immunochemical (12-14), and biochemical (10, 11, 16, 18) methods. The biochemical organization is only recently becoming clarified. The chain is branched, with each branch terminating in a cytochrome oxidase complex, denoted here as the cytochrome *d* complex and the cytochrome *o* complex. The cytochrome *d* complex, present under low aeration, contains three spectroscopically distinguishable entities, cytochromes *b*<sub>558</sub>, *a*<sub>1</sub>, and *d* (18). This complex has been shown to be a coupling site in *E. coli* and to function in vitro as a ubiquinol-8 oxidase in reconstituted proteoliposomes (J. Koland, M. Miller, and R. Gennis, *Biochemistry*, in press). The cytochrome *o* complex, present under high aeration, contains cytochromes *b*<sub>562</sub> and *b*<sub>555</sub> (11, 14, 16), which may actually be a single cytochrome with a split- $\alpha$  absorption band (14a). This complex has also been shown to be a coupling site in in vitro reconstitution experiments (10, 16). One other cytochrome, *b*<sub>556</sub>, has been isolated (11) and is present in the membranes regardless of which terminal oxidase is present.

A number of models have been proposed for the sequence of components in the aerobic respiratory chain (1). One point of disagreement among competing models is the placement of quinones in the electron transport sequence. For example, Haddock and Jones (6) suggest that quinones accept electrons from the dehydrogenases and can pass them along into either branch, in essence serving as the branch point between the two chains. Models suggested by Downie and Cox (3) and by Kita and Anraku (9) have quinone carrying electrons at two points, both before and after specific *b* cytochromes. These models are based primarily on data obtained by using ubiquinone-deficient mutants and comparing the steady-state level of cytochrome reduction in mutant strains with that in the parent.

It is the purpose of this work to reexamine these experiments by using strains which lack the cytochrome *d* terminal oxidase complex (4). The specific question is the location(s) of quinone in the branch of the aerobic respiratory chain terminating in cytochrome *o*. It is concluded that these

experiments provide no support for models in which quinone is required for the reoxidation of cytochromes in the respiratory chain.

### MATERIALS AND METHODS

**Bacterial strains.** The strains of *E. coli* K-12 which were used in this work are listed in Table 1. Strains GR66N, GR67N, GR68N, and GR69N were derived from strains AN387, AN386, AN385, and AN384, respectively, by transducing to tetracycline resistance with a P1 lysate grown on GR40N (*cyd*), which has Tn10 inserted near the *cyd* gene. The tetracycline-resistant transductants were scored spectroscopically for *cyd*.

**Media and growth of cells.** Cells (1 liter) were grown aerobically at 37°C in a 2-liter Klett flask containing, per liter, 100 ml of 10× minimal A medium (17), 10 g of glucose, 1 g of yeast extract, 0.15% Casamino Acids, 10 mg of thiamine-hydrochloride, with 1  $\mu$ M nicotinic acid, 1 mM MgSO<sub>4</sub> and 0.1 mM CaCl<sub>2</sub>. Cells were harvested in the early exponential phase by centrifugation at 8,000 rpm for 20 min.

Strains GR68N and GR69N had a tendency to revert back to *ubi*<sup>+</sup>, and the presence of revertants was checked by streaking samples of cultures on succinate minimal plates. Only those cultures which contained no revertants were used for subsequent experiments.

**Preparation of membranes.** For the preparation of membranes, cells were suspended (5 ml/g of cells) in 0.1 M potassium phosphate buffer, pH 7.0, and were disrupted with a Heat Systems W350 sonicator (50% pulse) for 3 min. Power was set at 20% of maximum. Whole cells were removed by centrifugation at 10,000 × *g* for 10 min. The membranes were collected by ultracentrifugation for 1 h at 45,000 rpm in a Beckman Ti60 rotor. The membrane pellet was then suspended in 0.1 M potassium phosphate buffer, pH 7.0, to a protein concentration of about 4 mg/ml.

**Spectrophotometric measurement.** Difference spectra were recorded at room temperature in an Aminco DW2 spectrophotometer. The wavelength pair employed was 560 and 575 nm, and the extinction coefficient used was 17.5 per mM per cm (2).

**Determination of steady-state oxidation-reduction levels.** Three-milliliter samples of membrane suspension (~4 mg of

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TABLE 1. Strains used<sup>a</sup>

Strain	Quinone characteristic	Genotype	Source or reference
AN384	<i>ubi men</i>	<i>ubiA420 menA401 rpsL gal</i>	26
AN385	<i>ubi men</i> <sup>+</sup>	<i>ubiA420 rpsL gal</i>	26
AN386	<i>ubi</i> <sup>+</sup> <i>men</i>	<i>menA401 rpsL gal</i>	26
AN387	<i>ubi</i> <sup>+</sup> <i>men</i> <sup>+</sup>	<i>rpsL gal</i>	26
GR40N	<i>ubi</i> <sup>+</sup> <i>men</i> <sup>+</sup>	<i>nadA::Tn10 cyd-1 thi rpsL</i>	This work
GR66N	<i>ubi</i> <sup>+</sup> <i>men</i> <sup>+</sup>	<i>nadA::Tn10 cyd-1 rpsL</i>	This work
GR67N	<i>ubi</i> <sup>+</sup> <i>men</i>	<i>nadA::Tn10 cyd-1 menA401 rpsL</i>	This work
GR68N	<i>ubi men</i> <sup>+</sup>	<i>nadA::Tn10 cyd-1 ubiA420 rpsL</i>	This work
GR69N	<i>ubi men</i>	<i>nadA::Tn10 cyd-1 ubiA420 menA401 rpsL</i>	This work

<sup>a</sup> The *gal*<sup>+</sup> genotype, along with *cyd-1* and *Tn10*, was transduced into strains GR66N through GR69N with P1 lysate grown on GR40N.

membrane protein per ml) were placed in sample and reference cuvettes (path length, 1 cm). Oxygen was slowly bubbled through the solution in the sample cuvette throughout the experiment, except during spectroscopic measurements. The sample in the reference cuvette was oxidized by adding a few grains of potassium ferricyanide. The reaction was initiated by injecting substrate into the sample cuvette. Substrates used were NADH (final concentration, 2 mM), DL-lactate (10 mM), and succinate (10 mM). The concentrations used were well above the  $K_m$  values (NADH, 0.15 mM; DL-lactate, 2 mM; succinate, 0.3 mM; M. Miller, personal communication). The reduction of cytochrome *b* in the aerobic steady state was expressed as a percentage of the total cytochrome *b*, as determined by dithionite reduction.

**Measurement of oxygen uptake.** The rate of oxygen uptake was measured with a Clark-type oxygen electrode with a YSI model 53 oxygen monitor. The reaction was carried out at 37°C in a chamber containing 1.8 ml of 0.1 M potassium phosphate buffer, pH 7.0. The substrates used were 4 mM NADH, 10 mM DL-lactate, and 10 mM succinate.

**Estimation of protein.** Protein concentration was measured by the method of Lowry et al. (15) with bovine serum albumin as a standard. The procedure was modified so that the final solution contained 1% dodecylsulfate.

## RESULTS

Four isogenic strains were constructed for these experiments: GR66N (*ubi*<sup>+</sup> *men*<sup>+</sup>), GR67N (*ubi*<sup>+</sup> *men*), GR68N (*ubi men*<sup>+</sup>), and GR69N (*ubi men*). Membranes from each strain were prepared, and the oxygen utilization rates with NADH, DL-lactate, and succinate are shown in Table 2. These values are in general agreement with those of Wallace and Young (26), who first compared the effects of ubiquinone and menaquinone deficiencies on the aerobic respiration rates exhibited by membrane preparations. The membranes examined by Wallace and Young (26) contained cytochrome *d*, whereas all the strains examined in these

TABLE 2. Oxygen utilization of *E. coli* membrane preparations

Substrate	Oxygen utilization in preparations from strain <sup>a</sup> :			
	GR66N ( <i>ubi</i> <sup>+</sup> <i>men</i> <sup>+</sup> )	GR67N ( <i>ubi</i> <sup>+</sup> <i>men</i> )	GR68N ( <i>ubi men</i> <sup>+</sup> )	GR69N ( <i>ubi men</i> )
Succinate	172	100	36	15
D L-lactate	240	129	40	10
NADH	495	800	53	30

<sup>a</sup> Measured as nanomoles of molecular oxygen per minute per milligram of membrane protein. Experimental details are provided in the text.

experiments were *cyd*, which results in the loss of cytochromes *b*<sub>558</sub>, *a*<sub>1</sub>, and *d*.

The steady-state levels of cytochrome reduction were determined with all four strains by using three different substrates. The data are presented as the percentage of the total cytochrome *b* which is reduced as a function of time after the addition of oxidizable substrate. During these experiments, oxygen was maintained at a saturating concentration by bubbling it through the cuvette continuously. Figure 1 shows the results obtained with strain GR66N (*ubi*<sup>+</sup> *men*<sup>+</sup>). After a few minutes, the steady-state reduction of the *b* cytochromes was observed, and it ranged between 5 and 25%, depending on the substrate. Typical room-temperature reduced-minus-oxidized spectra are shown in Fig. 2. Spectrum A was observed during the steady state with strain GR67N (*ubi*<sup>+</sup> *men*) with NADH as a reductant. Spectrum B was observed after the flow of oxygen was stopped and the sample was allowed to become anaerobic. The amount of reduced cytochrome clearly increased when the oxygen was exhausted.

The steady-state levels of cytochrome reduction with membranes from strain GR67N (*ubi*<sup>+</sup> *men*) were also examined. The menaquinone deficiency had little effect on the steady-state levels of cytochrome *b* reduction (data not shown). This is consistent with the fact that, although menaquinone can partially substitute for ubiquinone in the aerobic respiratory chain, it is not an essential component (26). It is important to note that the steady state could be maintained until the substrate (e.g., DL-lactate) was depleted, at which point all the cytochromes become oxidized. The addition of 20 mM lactate resulted in reattaining the same steady-state level of cytochrome reduction. This result indicates that a true steady state was obtained.

In Fig. 3, the results obtained with strain GR68N (*ubi men*<sup>+</sup>) are illustrated. With all three substrates, the levels of cytochrome reduced were lower than those observed with the isogenic strain GR66N (*ubi*<sup>+</sup> *men*<sup>+</sup>). In other words, ubiquinone deficiency caused by the *ubiA* allele decreased the amount of cytochrome reduced during the steady state. Figure 4 shows the effects of eliminating the oxygen flow through the system. For a few minutes, the steady-state level of cytochrome reduction was maintained at a low value, close to that observed when oxygen flow was maintained. After this period, the oxygen was exhausted, and there was a rapid increase in the level of cytochrome reduction. The

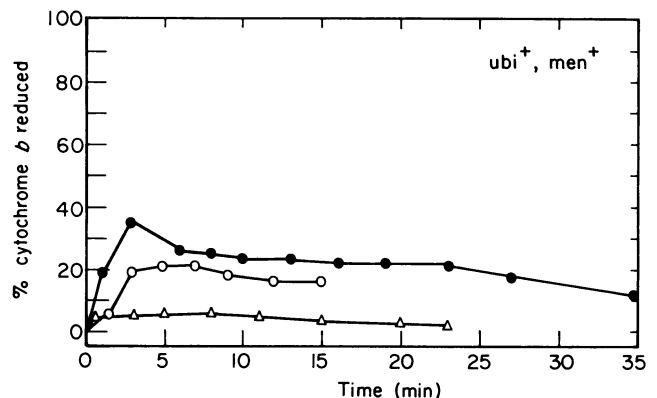


FIG. 1. Percentage of cytochrome *b* in the membranes of strain GR66N (*ubi*<sup>+</sup> *men*<sup>+</sup>) which was reduced after the addition of the oxidizable substrates 2 mM NADH (○), 10 mM succinate (△), and 10 mM DL-lactate (●). Experimental details are given in the text.

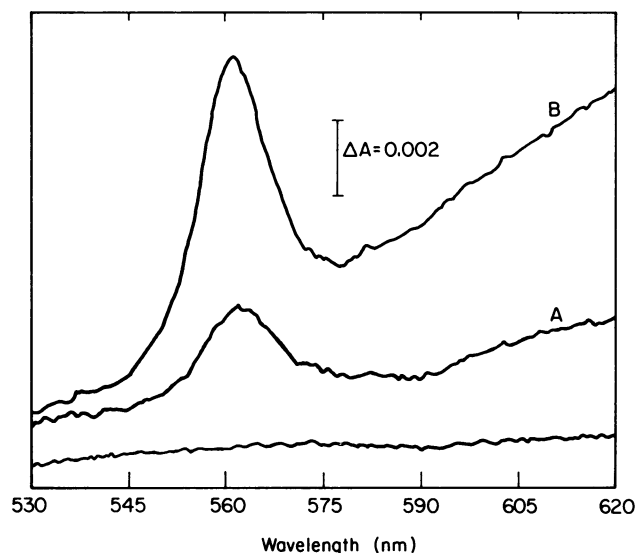


FIG. 2. Room-temperature difference spectra of membranes from strain GR67N (*ubi*<sup>+</sup> *men*) with 2mM NADH as substrate. (A) Steady-state-minus-oxidized difference spectrum. (B) NADH-reduced-minus-oxidized difference spectrum. After recording spectrum A, the oxygen flow was stopped, and oxygen was depleted after a few minutes. Spectrum B was then recorded. The base line is shown at the bottom.

length of time during which the steady state was maintained varied inversely with the oxidase activity observed with the substrates utilized. These data show that the steady-state levels of cytochrome reduction are not an artifact owing to oxygen flow, but rather that the oxygen flow merely extends the period of observation during which one can confidently examine the true steady-state properties of the system.

Membranes from strain GR69N (*ubi* *men*) were also examined (data not shown). Very little, if any, cytochrome reduction was observed. This clearly shows that cytochrome reduction is entirely dependent on the presence of quinones in the membrane.

#### DISCUSSION

The experiments reported in this work clearly show that the steady-state level of cytochrome *b* reduction is lower in strains which are lacking ubiquinone than in strains with no

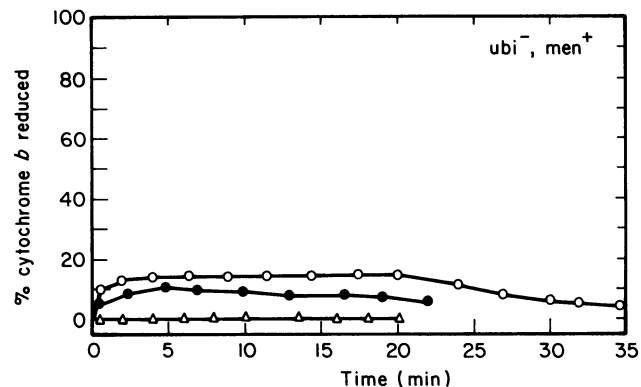


FIG. 3. Percentage of cytochrome *b* reduced in membranes from strain GR68N (*ubi*<sup>-</sup> *men*<sup>+</sup>) after the addition of the oxidizable substrates 2 mM NADH (○), 10 mM succinate (△) and 10 mM DL-lactate (●).

quinone deficiency. This contradicts the results reported by both Downie and Cox (3) and Kita and Anraku (9). One difference is that all the strains examined in this laboratory lacked the cytochrome *d* complex. This is probably not the source of the discrepancy, however. More likely, differences in the experimental protocols for attaining the steady state are responsible for the different results obtained. In this work, the oxygen concentration was maintained by passing oxygen gas through the cuvette. It was demonstrated that a true steady state was obtained and could be maintained as long as the substrate was replenished. In the absence of oxygen flow, the samples became anaerobic in just a few minutes, and there was a sharp rise in the level of cytochrome reduction. The procedure used by Downie and Cox (3) was to place the sample in a cuvette (path length, 2 mm) used with the Aminco DW2 low-temperature accessory. After 30 s at room temperature, the samples containing the wild-type membranes were frozen, and the spectra were

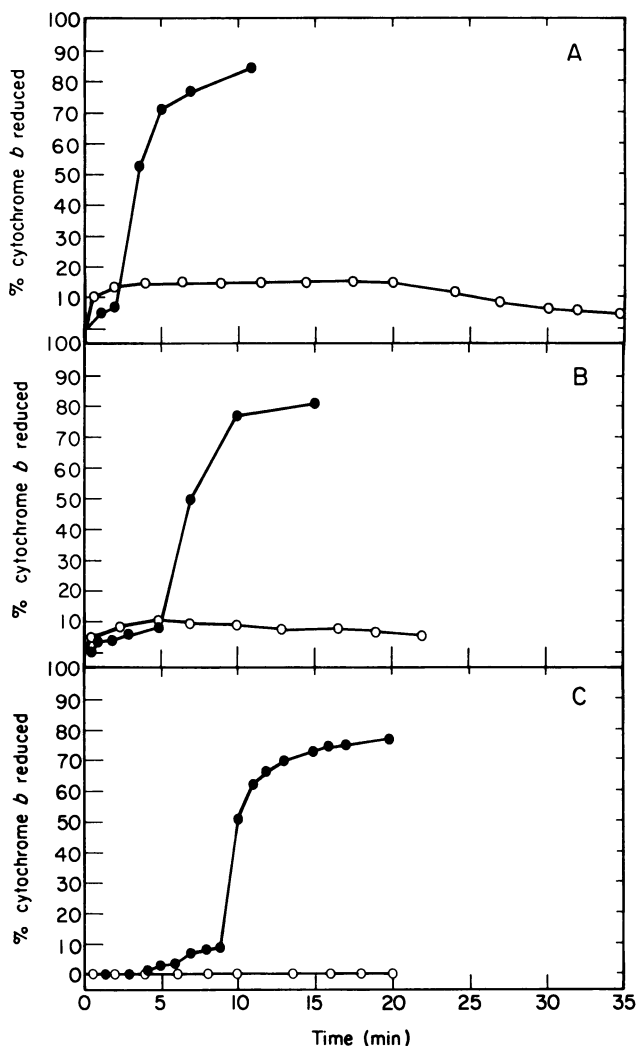


FIG. 4. Comparison of the percentages of cytochrome *b* reduced in the presence (○) and absence (●) of oxygen flow through the cuvette. Membranes from strain GR68N (*ubi* *men*<sup>+</sup>) were used, as in Fig. 3. Substrates were 2 mM NADH (A), 10 mM DL-lactate (B), and 10 mM succinate (C). The abscissa shows the time after the addition of the substrate to the cuvette. Full reduction (100%) is the value obtained with dithionite as the reductant.

recorded at 77 K. For the membranes from the ubiquinone-deficient strain, the samples were incubated for 180 s before being frozen. It was claimed that it took longer for these samples to attain the steady-state level of cytochrome *b* reduction than for those from the wild-type strain. It is quite likely that these samples were not in the steady state but were rather depleted of oxygen, resulting in high levels of cytochrome reduction. Oxygen depletion would occur quite rapidly in a cuvette with a path length of 2 mm since the rate of oxygen diffusion into the solution would be smaller than in larger cuvettes used in the work reported here. Kita and Anraku (9) do not provide enough experimental details to explain the discrepancy between their results and ours, but a similar explanation probably applies in their case as well. It should be noted that although both Downie and Cox (3) and Kita and Anraku (9) report increased cytochrome reduction in the steady state in the *ubi* strain, they disagree substantially about which cytochromes are reduced.

The data in this paper show that there is no experimental basis for postulating a role for quinone in the aerobic respiratory chain after any cytochrome. The results are consistent with a model in which a quinone pool is responsible for carrying electrons from the dehydrogenases to the cytochromes of either branch of the respiratory chain. It has been directly demonstrated in reconstituted proteoliposomes that the cytochrome *d* complex functions as a ubiquinol-8 oxidase (Koland et al., *Biochemistry*, in press). The organization of the branch terminating in cytochrome *o* is less clear. Cytochromes *b*<sub>555</sub> and *b*<sub>562</sub> are clearly part of a single complex (11, 14, 16). If cytochrome *b*<sub>556</sub> is actually required for the respiratory chain, which has yet to be demonstrated, then it would be needed for transferring electrons to the cytochrome *o* complex, either directly or through another electron carrier. The results from this work do not rule out the possibility that ubiquinone is required for electron transfer between cytochrome *b*<sub>556</sub> and the cytochrome *o* complex (9). To resolve this portion of the electron transport chain, further experiments will be required.

#### ACKNOWLEDGMENTS

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