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Lipophilic Chelator Inhibition of Electron Transport in *Escherichia coli*

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The lipophilic chelator bathophenanthroline inhibits electron transport in membranes from *Escherichia coli*. The less lipophilic 1,10-phenanthroline, bathophenanthroline sulfonate, and α,α-dipyridyl have little effect. Reduced nicotinamide adenine dinucleotide oxidase is more sensitive to bathophenanthroline inhibition than lactate oxidase activity. Evidence for two sites of inhibition comes from the fact that both reduced nicotinamide adenine dinucleotide menadione reductase and duroquinol oxidase activities are inhibited. Addition of uncouplers of phosphorylation before bathophenanthroline protects against inhibition.

On the basis of studies with coenzyme Q-deficient mutants and electron spin resonance studies, Gibson and co-workers (3, 4) have proposed that there are two centers for coenzyme Q and iron-sulfur protein function in the electron transport system of *Escherichia coli*. Kurup and Brodie (7) have shown that the chelator 1,10-phenanthroline inhibits phosphorylation and malate oxidation in *Mycobacterium phlei* as evidence of a metalloprotein function. In these studies it has been found that the more lipophilic chelator 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline [BP]) acts as an excellent inhibitor of electron transport in *E. coli*. By introducing artificial electron donors to explore partial reaction sequences within the overall electron transport chain, it has been possible to develop a new line of evidence that there can be at least two non-heme iron centers in the chain.

MATERIALS AND METHODS

Preparation of E. coli membranes. The growth medium selected was medium 56 described by Monod et al. (8) and modified by Newton et al. (9) as: 10.6 g of K_2HPO_4, 6.1 g of NaH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of $(NH_4)_2SO_4$, 10 mg of $Ca(NO_3)_2$, and 0.5 mg of $FeSO_4 \cdot 7H_2O$ dissolved in 1 liter of water. Glucose was sterilized separately and added to a final concentration of 30 mM.

Colonies of *E. coli* K-12 were selected from streak plates and used to inoculate tubes containing 10 ml of the glucose-salts medium. These tubes were incubated overnight at 37 C and were then used to inoculate about 700 ml of the same medium in 2-liter flasks (20 ml of inoculum per flask). The inoculated flasks were incubated overnight at 37 C on a shaker. Shaking was vigorous to allow the maximum practical aeration of the cultures.

Cells were harvested by chilling the flasks in ice and centrifuging at 8,000 × *g* for 20 min. This was followed by washing the cells in cold 0.25 M sucrose-0.1 M phosphate buffer (pH 7.0) and collection again by centrifugation at 8,000 × *g* for 20 min. The cells were resuspended in an equal volume of sucrose-phosphate buffer.

Disruption of the cells was accomplished by sonic treatment with a Bronson Sonifier tuned to 8 to 9 A. Sonic treatment was in eight bursts of 30-s duration while the cells were chilled in ice. The sonically treated mixture was then centrifuged at 27,000 × *g* for 20 min to sediment cells that were not sonically treated and cell wall debris, and the resulting supernatant was centrifuged at 100,000 × *g* for 2.5 h. The gelatinous yellow pellet from this final centrifugation was suspended and homogenized in a minimal volume of 0.1 M phosphate buffer (pH 7.0) and designated the "membrane fraction." Protein concentration was determined by the biuret procedure (14).

Assay of oxidase activities, as evidenced by uptake of oxygen, was measured polarographically with an oxygen electrode. Unless otherwise stated, the membrane suspension was adjusted to a concentration of 8 to 10 mg of protein per ml. All assays were performed in 1.4 ml of 0.1 M phosphate buffer (pH 7.0) at 27 C.

For the reduced nicotinamide adenine dinucleotide (NADH) oxidase assay, 50 μl of membrane suspension was incubated in buffer for 3 min in the presence or absence of the indicated amounts of inhibitors and uncoupler. A 50-μl volume of 0.01 M NADH was then added to start the reaction.

Lactate oxidase was assayed in the same manner as for NADH oxidase, except that 100 μl of membrane suspension was used, and the reaction was started by addition of 50 μl of 0.1 M lactate.

Duroquinol oxidase assays were performed by incubation of 100 μl of membrane suspension in buffer in the presence or absence of inhibitors and uncouplers for 3 min. To start the reaction, 15 μl of duroquinone (20 mg/ml in ethanol) was added, followed immediately by addition of 25 μl of dithiothreitol (30 mg/ml).

Tetrachloroquinol oxidase was assayed as for duroquinol oxidase except that 10 μl of tetrachloroquinol (3 mg/ml in ethanol) was substituted for duroquinol.

NADH-menadione reductase activity was measured by incubation of 25 μl of membrane suspension (5 mg of protein/ml) and 30 μl of KCN (0.1 M) in buffer in the presence or absence of inhibitors and uncoupler for 3 min. The reaction was started by addition of 25 μl of menadione (75 mM in ethanol) followed immediately by addition of 50 μl of NADH (0.01 M). To determine the hydroxyquinoline-*N*-oxide (HOQNO)-insensitive rate, 25 μl of membrane suspension, 30 of μl KCN, and 10 μl of HOQNO (4.0 mg/ml in ethanol) were incubated with the appropriate inhibitors and uncouplers, and the reaction was begun as described above.

RESULTS

BP inhibits both NADH and lactate oxidase activity of *E. coli* membranes (Fig. 1). NADH oxidase is inhibited 50% at a BP concentration of 5×10^{-5} M. Lactate oxidase shows 50% inhibition only at 1.3×10^{-4} M and is saturated at that concentration. The oxidation of duroquinol is inhibited by BP 50% at 10^{-4} M in a pattern which is similar to the inhibition shown for lactate oxidase.

As with NADH and lactate oxidase (3), the oxidation of duroquinol is inhibited by 2-heptyl-4-HOQNO, coenzyme Q analogues 5-ω-cyclohexyl pentyl-6-hydroxy-2,3 dimethoxy-1,4-benzoquinone, and 7-*n*-pentadecyl-6-hydroxy-5,8-quinolinequinone (2). When added to *E. coli* membranes in the presence of KCN, duroquinol causes the reduction of a *b* type cytochrome with α band absorbance maximum at 562 nm. Subsequent addition of lactate causes the additional reduction of a *b* component with a maximum at 560 nm (Fig. 2).

NADH oxidase shows only slight inhibition by the uncoupler carbonylcyanide-*m*-chlorophenyl hydrazone (CCCP) (Fig. 3). In the presence of CCCP there is a consistent decrease of BP inhibition of 10 to 40% (Fig. 3).

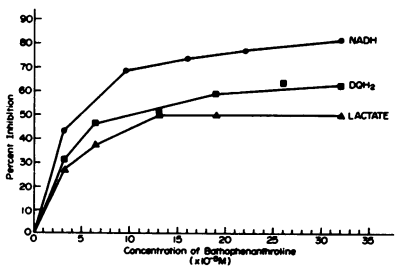

FIG. 1. *Inhibition of NADH, lactate, and duroquinol oxidase activity by BP. Uninhibited activity was: NADH oxidase, 457; lactate oxidase, 92; and duroquinol oxidase, 184 nmol of O_2/min × mg of protein. At 0.5×10^{-3} M, BP sulfonate gave 12% inhibition and α,α-dipyridyl and orthophenanthroline gave no inhibition of NADH oxidase.*

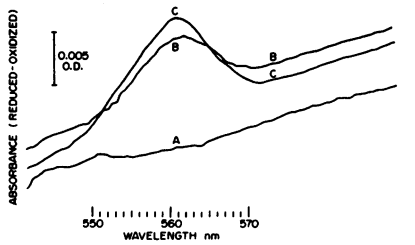

FIG. 2. *Difference spectrum of E. coli membrane: (A) 10 mg of membrane preparation in each cuvette, (B) 5 μmol of duroquinone and 20 μmol of dithiothreitol added to the sample cuvette; (C) 50 μmol of DL-lactate added to sample cuvette after 5 μmol of duroquinone and 20 μmol of dithiothreitol.*

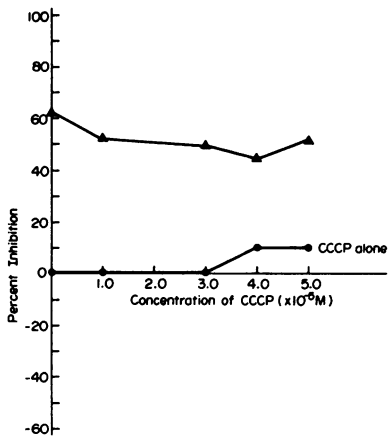

FIG. 3. *Effect of CCCP and BP on NADH oxidase. Uninhibited oxidase rate 354 nmol of O_2/min × mg of protein. Rates with BP corrected for CCCP stimulation.*

Menadione shows excellent activity as an alternative acceptor for the oxidation of NADH. The activity is 50% inhibited by HOQNO (Table 1). BP inhibits the overall menadione reduction rate up to 33%, most of which can be accounted for as inhibition of the HOQNO-sensitive rate (59%) since the menadione reduction in presence of HOQNO is only 15% inhibited. The BP inhibition of the HOQNO-sensitive rate is decreased by prior addition of CCCP to the membranes, whereas there is no effect of CCCP on the BP inhibition of the HOQNO-insensitive rate (Fig. 4). It should be noted that CCCP alone shows a stimulation of the NADH menadione reduction activity in contrast to minor effects on NADH oxidase.

Lactate oxidation is inhibited by CCCP (Fig. 5) and, in contrast to NADH oxidase, the BP inhibition of lactate oxidase shows a steady decrease with increasing concentration of CCCP

TABLE 1. *Effect of BP on NADH menadione reductase activity in the presence and absence of heptyl HOQNO*

BP concn ($\times 10^{-5}$ M)	NADH menadione reductase activity		HOQNO-sensitive activity (by difference)	
	No addition (A)	Plus 40 μg of HOQNO (B)	(A - B)	% Inhibition
0	917[a]	554[a]	363[a]	0
3.2	786	513	273	25
6.3	689	513	176	52
12.8	655	459	196	46
19.0	614	466	148	59

[a] Rate in nanomoles of O_2 per minute $\times$ milligram of protein.

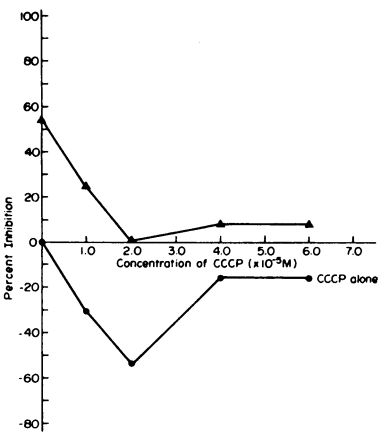

FIG. 4. *Effect of CCCP on the HOQNO-sensitive NADH menadione reductase activity. Symbols: ●, effect of CCCP alone; ▲, effect of BP ($\times 10^{-5}$ M) in presence of CCCP.*

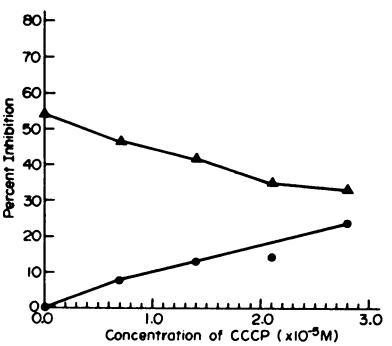

FIG. 5. *Effect of CCCP and BP on lactate oxidase. Symbols: ●, inhibition by CCCP alone; ▲, inhibition by 1.3 $\times 10^{-4}$ M BP in presence of CCCP. Uninhibited oxidase rate 107 nmol of O_2/min $\times$ mg of protein.*

so that the maximum reversal is at higher concentrations than with NADH oxidase.

Duroquinol oxidase is inhibited 60% by 10 $\times$ 10^{-5} M BP (Fig. 6). This inhibition is 25% less in presence of 4 $\times$ 10^{-5} M CCCP. The inhibition of duroquinol oxidase by CCCP is similar to the inhibition of lactate oxidase by both coenzyme Q analogues and BP.

The oxidation of tetrachloroquinol, which has a much higher redox potential (E'_0 pH 7.0 =

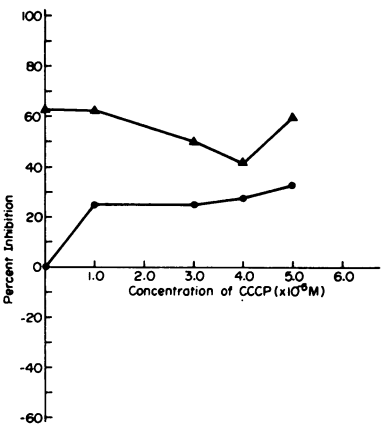

FIG. 6. *Effect of CCCP and BP on duroquinol oxidase activity. Symbols: ●, inhibition by CCCP alone; ▲, inhibition by 1.3 $\times 10^{-4}$ M BP in presence of CCCP.*

TABLE 2. *Comparison of the effect of inhibitors on duroquinol and tetrachloroquinol oxidase activities*

Addition	Relative oxidation rate[a]	
	Duroquinol oxidase	Tetrachloroquinol oxidase
None	100	100
HOQNO (10 μg)	0	93
HOQNO (4 μg)	27	
BP (2.0×10^{-4} M)	37	30
BP (2.0×10^{-4} M) + CCCP (4.0×10^{-5} M)	57	32
2,5-Dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (1.5 nmol)[b]	64	120
5-ω cyclohexylpentyl-6-hydroxy-2,3-dimethoxy benzoquinone (25 μg)	7	85
7-nhexadecylmercapto-6-hydroxy-5,8-quinolino quinone (15 μg)	19	100
3-ndodecylmercapto-2-hydroxy-1,4-naphthoquinone (3 μg)	7	120

[a] Control rates: Duroquinol oxidase 0.11 μmol/min $\times$ mg of protein; tetrachloroquinol oxidase 0.047 μmol/min $\times$ mg of protein at 30 C.

0.29 V) than duroquinol, shows very little inhibition by the coenzyme Q analogues, indicating a site of oxidation beyond either proposed coenzyme Q site. A chelator-sensitive site in the terminal portion of the electron transport chain is indicated by the strong BP inhibition. Since this inhibition is not decreased by CCCP, it appears to be different from other BP sites (Table 2).

DISCUSSION

The results can be best considered in relation to the previous proposal for electron transport in *E. coli* of Cox et al. (3). They propose a non-heme iron–coenzyme Q complex in the segment between NADH dehydrogenase and cytochrome *b*. This could be the most sensitive BP site, which shows a weak response to CCCP. This site can also be the site where the HOQNO-sensitive reduction of menadione occurs, with the menadione reaction more closely related to the portion where CCCP gives a stronger reversal of BP inhibition. The weak BP inhibition of the HOQNO-sensitive activity indicates a chelator-sensitive site closer to the primary dehydrogenase. The existence of several non-heme iron sites in this portion of the electron transport chain would not be surprising in view of the numerous sites found associated with dehydrogenases in mitochondria (6, 10; I. Sun, D. C. Phelps, R. T. Crane, and F. L. Crane, Proc. Indiana Acad. Sci., in press).

Duroquinone appears to be oxidized through the segment of the chain from cytochrome *b* to oxygen. This is based on the following: (i) the redox potential E'_0 (pH = 7.0) for the duroquinone couple is +0.05 V; (ii) duroquinol reduces a cytochrome *b;* (iii) duroquinol is oxidized in the cytochrome *b* region in mitochondria (5); (iv) duroquinol oxidation is inhibited by HOQNO and by coenzyme Q analogues. This would be consistent with a second non-heme iron–coenzyme Q site which is HOQNO sensitive between cytochrome *b* and the terminal oxidase as proposed by Cox et al. (3). The BP inhibition is consistent with a non-heme iron site.

The effect of BP on lactate oxidation could appear to be very similar to the effect of BP on duroquinol oxidation and the protective effect of CCCP gradually increases up to 4×10^{-5} M. The BP inhibition of both of these activities can most readily be related to a non-heme iron site between cytochrome *b* and oxygen. Whether there are other chelator-sensitive sites in lactate oxidation will require further development of partial assays for the lactate to cytochrome *b* segment. The lack of CCCP effect on NADH oxidase can be the sum of a stimulatory effect in the first segment and an inhibition in the terminal part of the chain.

The chelator-sensitive site seen in tetrachloroquinol oxidase appears to be beyond any site of coenzyme Q function. It should be noted that there is no uncoupler protection against BP inhibition of tetrachloroquinol oxidase activity.

The phenomenon of uncoupler protection against BP inhibition has been observed to be correlated with coupling sites in both mitochondria (12) and chloroplasts (1). From these studies it appears that similar sites exist in the *E. coli* membrane. Walker and Mortenson have also shown that adenosine 5′-triphosphate causes iron in azoferridoxin to be available to chelator attack (13), indicating that the energized state involves a conformation in which iron is exposed. It should also be noted that the Mg^+-stimulated membrane-bound adenosine triphosphatase of *E. coli* is inhibited by BP and that uncoupler protect against this inhibition. Since one-half maximum BP inhibition of adenosine triphosphatase is seen at a concentration similar to that required to inhibit NADH oxidase or duroquinol oxidase (7.2×10^{-5} M), the adenosine triphosphatase inhibition gives further evidence that coupling sites are involved in this inhibition (11; Sun et al., in press).

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