LIPOPHILIC CHELATOR INHIBITION OF *ESCHERICHIA COLI* MEMBRANE-BOUND ATPase ACTIVITY AND PREVENTION OF INHIBITION BY UNCOUPLERS

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1. Introduction

It has been well documented that the ATPase activity is associated with oxidative phosphorylation. Lately, it has become obvious that the enzyme is normally part of the biological membrane and is the terminal enzyme in the energy coupling reactions [1–3].

Evidence for the participation of non-heme iron proteins as functional components in the electron transport chain has been firmly established, but there is little evidence for the direct involvement of these proteins in energy coupling or phosphorylation. In electron transport systems from Mycobacterium-phlei and rat liver mitochondria Kurup et al. [4] and Palmer [5] respectively have shown that the uncoupling effect of metal chelating agents can be reversed by adding ferrous ion. From the study of beef heart submitochondrial particles, Vallin et al. [6,7] and Butow and Racker [8] proposed models for respiratory control correlated to oxidative phosphorylation through non-heme iron proteins. Recently, Phelps et al. [9,10] have presented experimental evidence that metalproteins act as common functional intermediates for both electron transport and energy conserving reactions of beef heart mitochondria.

Bathophenanthroline (4,7-diphenyl-1,10-phenanthroline), which is a lipophilic metal chelating agent also provides a useful tool for the study of the same phenomenon in a prokaryotic system. In this paper we show that bathophenanthroline inhibits *E. coli* membrane bound ATPase activity as completely as that of eukaryotic organelles and at similar concentration. Uncouplers reverse the inhibition. This kind of inhibition is not observed in high Mg^{++} buffer with the soluble ATPase. Since bathophenanthroline has a high affinity for ferrous iron, we propose that a buried non-heme iron protein functions at a site in the membrane which controls the activity of the ATPase and therefore suggest the direct involvement of this protein in the energy conserving system.

The experiments of Abrams and Baron [11] suggest that the potency of *N,N'*-dicyclohexylcarbodiimide (DCCD) is due to the ability of DCCD to penetrate a highly hydrophobic environment. This together with the fact that the DCCD binding protein (~10 000 dalton) is a proteolipid [12], indicates that this protein is normally also deep buried in the membrane. Since our results show that DCCD does not reverse bathophenanthroline inhibition, that the uncoupler carbonyl cyanide-3-chlorophenyl hydrazone (CCCP) does not reverse DCCD inhibition, and DCCD inhibits the ATPase activity restored by CCCP in the presence of bathophenanthroline, we conclude that the DCCD-binding protein is different from the bathophenanthroline sensitive component.

2. Materials and methods

The medium used for the growth of *E. coli* strain K12 wild type was that described by Monod et al. as medium 56 [13]. The strain AN236 (ilv^-, arg^-, purE, thi^-), AN283 (arg^-, $entA^-$, $uncb^-$), AN259 (arg^-, $entA^-$) and AN293 (arg^-, thi^-, $ubiB^-$) were provided by Dr F. Gibson and were grown in a manner des-

cribed by Cox et al. [14]. The appropriate L-amino acids were added to the sterilized mineral-salt base to give a final concentration of 0.2 mM, thiamine and adenine were added to a final concentration of 0.2 μM. The carbon source was added as a sterile solution at a final concentration of 30 mM. A freshly prepared slant was used to inoculate 700 ml of the same medium in 2 liter flasks which are then incubated overnight at 37°C on a rotary shaker.

Cells were harvested by centrifugation at 27 000 *g* for 10 min, washed in cold 0.25 M sucrose–0.1 M phosphate buffer pH 7.0 and collected again by centrifugation at the same speed, the suspended in sucrose-phosphate buffer (0.5 g cell/ml.).

Membrane fraction was prepared by disruption of cells by sonication with a Bronson sonifier at 7–9 A for 3 min in ice. The cell extract was centrifuged at 27 000 *g* for 20 min and the supernatant fraction was then centrifuged at 100 000 *g* for 2 hr. The resulting gelatinous yellow pellet was suspended in a minimal volume of 0.1 M phosphate buffer pH 7.0. This suspension was designed as the membrane fraction.

Soluble ATPase was made according to Cox et al. [14] by low ionic stength treatment with more extensive washings. Trypsin treatment was performed as described by Nieuwenhuis et al. [15].

Protein was determined by the biuret method [16] with bovine serum albumin as a standard.

The ATPase activity was measured according to Phelps and Crane [9] at 30°C. This assay contains 0.5 mM ATP and 11.5 mM Mg^{++}. For low Mg^{++} conditions the assay contained 0.1 M Tris–HCl, 2 mM ATP, 2 mM $MgCl_2$, 1 mM DTT, pH 8.9. P_i was measured by the Lindberg and Ernster procedure [17].

3. Results and discussion

Bathophenanthroline, a lipophilic chelator inhibits *E. coli* K12 membrane bound ATPase activity as shown in fig.1. 40 μg (0.12 μmol) of bathophenantroline is able to cause maximum inhibition (70%). This concentration is higher than found for mitochondria where 20 μg (0.06 μmol) gives maximum inhibition [9]. The chelator specificity is shown in table 1. Orthophenanthroline (1 μmol) and α,α′-dipyridyl (0.26 μmol) show less than 10% and 15% inhibition respectively. Hydrophilic chelators such as

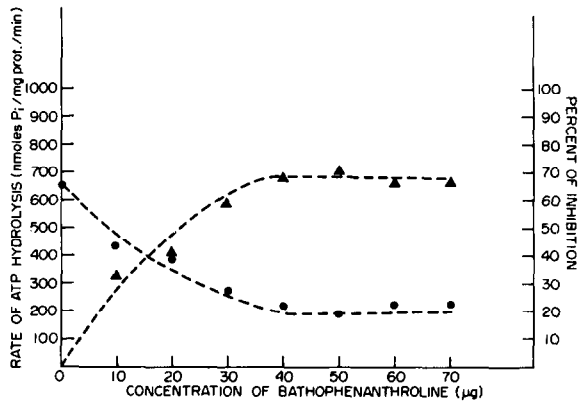

Fig.1. The inhibition of *E. coli* K12 membrane bound ATPase activity by bathophenanthroline. (●) Rate of ATP hydrolysis (nmoles P_i/mg protein/min); (▲) percent of inhibition. Protein concentration was 0.37 mg for the experiment.

bathophenanthroline sulfonate show essentially no inhibition. The complete lack of effects with bathophenanthroline sulfonate, and minor effects with orthophenanthroline and α,α′-dipyridyl indicate that the bathophenanthroline sites or the coupling sites are protected by a hydrophobic environment. The lack of a dithizone site contrasts with mitochondria where dithizone, a Cu chelator, inhibits cytochrome oxidase [10]. 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTFA), a powerful metal-chelating agent, has been shown to be a potent inhibitor of succinate oxidation in mitochondria [18], the mechanism of

Table 1
The inhibition of *E. coli* K12 membrane-bound ATPase activity by various chelating agents

Chelating agents	Percent of inhibition
0.12 μmol Bathophenanthroline	70
0.77 μmol Dithizone	0
0.37 μmol Bathophenanthroline sulfonate	0
1 μmol Orthophenanthroline	9.2
0.26 μmol α,α′-dipridyl	13.2
0.045 μmol TTFA	31
0.2 μmol FTFA	32

TTFA, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione.
FTFA, 4,4,4-trifluoro-0-(2-furyl)-1,3-butanedione.
Protein concentration was 0.17 mg per assay.

Table 2
The reversal of *E. coli* ATPase activity inhibited by bathophenanthroline with carbonyl cyanide-3-chlorophenyl hydrazone (CCCP)

Strain	Status of ATPase	Bathophenanthroline (μmoles/assay)	CCCP (nmoles/assay)	ATPase activity (μmoles/mg prot./min)
K12 W.T.	Membrane-bound	0	0	1.0
		0	14.4	0.8
		0	24.0	0.7
		0.03	0	0.7
		0.03	24.0	1.0
		0.09	0	0.5
		0.09	14.4	0.7
		0.09	24.0	0.9
		0.15	0	0.3
		0.15	24.0	0.6
		0.18	0	0.3
		0.18	14.4	0.9
		0.18	24.0	0.6
AN293	Membrane-bound	0	0	1.0
		0.15	0	0.6
		0.15	14.4	1.1
AN293	Soluble	0	0	1.0
		0.15	0	1.1
		0.15	14.4	1.1
AN293	Soluble trypsin treated	0	0	1.9
		0.15	0	1.8
		0.15	14.4	1.8
AN259 uncB^{+}	Membrane-bound	0	0	1.0
		0.15	0	0.6
		0.27	0	0.5
AN283 uncB^{-}	Membrane-bound	0	0	1.0
		0.15	0	0.9
		0.27	0	0.9

Protein concentration was 0.185 mg for strain K12 W.T., 0.29 mg for strain AN293 membrane bound enzyme, 0.17 mg for strain AN293 soluble enzyme (both trypsin treated and untreated) and 0.25 mg for both strain AN259 and AN283 per assay.

inhibition is assumed to be a chelation of non-heme iron. 4,4,4-trifluoro-1-(2-furyl)-1,3-butanedione (FTFA), an analog of TTFA is seen to inhibit mitochondrial electron transport reactions in complexes I, II and III [19]. The interference with non-heme iron sites by this inhibitor is also suggested. Since *E. coli* K12 membrane bound ATPase activity is inhibited by both TTFA and FTFA at a low level of these inhibitors we propose that a non-heme iron protein is a functional component in the energy coupling system. Bathophenanthroline does not inhibit the soluble ATPase both trypsin treated and untreated [15] as shown in table 2 in high Mg^{++} buffer. In low Mg^{++} a site for bathophenanthroline inhibition appears in the soluble enzyme whereas the membrane bound enzyme is less sensitive.

Table 2 shows the bathophenanthroline inhibition of membrane bound ATPase activity is reversed by

the uncoupler, CCCP. CCCP alone causes slight inhibition. With both CCCP and bathophenanthroline in the assay CCCP reverses the inhibition of bathophenanthroline. When bathophenanthroline concentration is low (0.03 μmol), 24 nmol of CCCP gives a complete reversal. With high bathophenanthroline concentration (0.15 μmol or 0.18 μmol), 24 nmol of CCCP gives only 60% reversal in the strain K12 wild type. This implies that there are several bathophenanthroline sensitive sites in the membrane sector; low bathophenanthroline inhibits primarily at those sites in the coupling region, at higher bathophenanthroline concentration, the inhibition of other sites, not in the coupling region may become significant which are not CCCP reversible. The sensitivity to bathophenantroline varies in different strains. 40% inhibition is observed in the strain K12 wild type at 0.06 μmol bathophenantroline; but 0.15 μmol and 0.27 μmol is required for the same inhibition in strain AN293 and AN259 respectively. AN283, an isogenic strain of AN259, deficient in uncB protein, is essentially insensitive to bathophenanthroline inhibition as shown in table 2. This indicates that the defect in uncB protein which is part of the membrane sector [20] somehow affects the response to bathophenanthroline. Other uncouplers also reverse the bathophenanthroline inhibition of ATPase as shown in table 3. Ionophores such as gramicidin, valinomycin and nigericin are essentially ineffective in reversing the bathophenanthroline inhibition of ATPase activities. The lack of reversal of bathophenanthroline inhibition of electron transport activities by ionophores is also observed in mitochondria [10]. Since ionophores which are known to prevent a proton gradient across the membrane do not bring the reversal of bathophenanthroline inhibition, we suggest that the ion gradients cannot be the primary step in energy transfer.

The preformed ferrous-bathophenanthroline, shows no more than 10% inhibition of ATPase activity in both membrane-bound and solubilized form. CCCP does not induce an increase of activity. This lack or inhibition is in contrast to mitochondria where the chelate gives up to 70% inhibition of ATPase activity and CCCP causes up to 50% reversal compared to the reversal of bathophenanthroline inhibition [9]. A different mechanism of inhibition by ferrous-bathophenanthroline is indicated in mitochondria which is missing in *E. coli*. The slight inhibition observed may be caused by a small amount of bathophenanthroline which is dissociated from ferrous-bathophenanthroline complex in the membrane.

The inhibition by DCCD of soluble ATPase activities at high concentration and membrane-bound ATPase activities at low concentration is shown in table 4. This is in contrast to Evan's result [21] where he shows that the solubilized form of *E. coli*

Table 3
Prevention of bathophenanthroline inhibition of *E. coli* K12 membrane-bound ATPase activity by various uncoupling agents

Uncoupling agents	ATPase activity (μmoles P_i/mg prot./min)		
	0 μmol Bathophenanthroline	0.09 μmol Bathophenanthroline	0.15 μmol Bathophenanthroline
No uncoupling agent	1.0	0.5	0.3
14.4 nmol CCCP	0.8	0.7	0.9
3.1 nmol TTFB	0.9	0.9	0.9
2.7 nmol S_6	0.4	0.9	0.8
2.5 nmol Pentachlorophenol	1.0	0.8	0.7
1.9 nmol Dicumarol	1.0	1.0	0.9
20 nmol Gramicidin + 100 μmoles potassium acetate	0.8	0.5	0.3
10 mol valinomycin + 14 μmol Nigericin + 100 μmol potassium	0.9	0.5	0.3

Protein concentration was 0.185 mg per assay
S_6, 5-chloro-3-(*p*-chlorophenol)-4'-chlorosalicylanilide, CCCP, carbonyl cyanide-3-chlorophenyl hydrazone; TTFB, 4,5,6,7 tetrachloro-2-trifluoromethylbenzimidazole.

Table 4
Effects of DCCD on ATPase activities of *E. coli* AN236 in the presence of CCCP, bathophenanthroline and both

μmoles Batho-phenanthroline	nmoles CCCP	nmoles DCCD	ATPase activity membrane-bound form	(μmoles P_i/mg prot/min) solubilized form
0	0	0	1.0	1.0
0	0	5.0	0.5	1.0
0	0	10.0	0.6	0.8
0	24.0	5.0	0.5	–
0	24.0	10.0	0.6	–
0.03	0	0	0.7	–
0.09	0	0	0.6	–
0.03	0	5.0	0.7	–
0.09	0	5.0	0.6	–
0.03	24.0	0	1.0	–
0.09	24.0	0	1.0	–
0.03	24.0	5.0	0.8	–
0.09	24.0	5.0	0.8	–

Protein was 0.10 mg for soluble ATPase and 0.11 mg for membrane-bound ATPase per assay.

ATPase has higher sensitivity to DCCD than the membrane bound form. DCCD is a known inhibitor of mitochondrial membrane bound ATPase [22] which causes a non-specific inhibition with various protein functional groups such as -COOH, try-OH, $-NH_2$, -SH and Ser-OH [23–26]. Since Evan applies a much higher DCCD concentration ($5–10 \times 10^{-5}$ molar) than we do ($2.5–5 \times 10^{-6}$ molar), the inhibition of the soluble ATPase can be a non-specific inhibition due to greater exposure of protein functional groups. That CCCP does not reverse DCCD inhibition of ATPase activities, that bathophenanthroline inhibition of ATPase activities is not reversed by DCCD and that DCCD inhibits ATPase activities reversed by CCCP in the presence of bathophenanthroline are also shown in table 4. Lower sensitivity of ATPase to DCCD inhibition in the presence of both CCCP and bathophenanthroline in comparison to DCCD alone may indicate a conformational change of ATPase complexes affected by CCCP and bathophenanthroline.

Under low Mg^{++} assay conditions the extent of inhibition of the membrane-bound ATPase is decreased and a partial inhibition of the soluble ATPase appears. Under these conditions CCCP increases the extent of inhibition of the membrane bound enzyme and decreases the extent of inhibition of the soluble enzyme (table 5). A similar inhibition of the soluble mitochondrial ATPase by bathophenantroline and decrease in extent of inhibition in

Table 5
Inhibition of soluble and membrane-bound ATPase activity by bathophenanthroline and bathophenanthroline sulfonate under low Mg^{++} assay conditions

Enzyme state	Inhibitor	Percent inhibition 11.5 mM Mg^{++}	2 mM Mg^{++}
Membrane-bound	BP 0.09 μmol	50	17
	BP 0.15 μmol	70	26
	BP 0.15 μmol + CCCP 14 nmol	10	41
	Bps 0.12 μmol	0	0
	Bps 0.6 μmol	0	–1
Soluble	BP 0.18 μmol	0	21
	BP 0.18 μmol + CCCP 24 nmol	0	7
	Bps 0.12 μmol	–	20
	Bps 0.6 μmol	–	34

Bp: 4.7-Diphenyl-1.10-phenanthroline.
Bps: 4.7-Diphenyl-1.10-phenanthroline sulfonate.

the presence of uncouplers under low Mg^{++} conditions has been reported by Phelps et al. [27]. We find, in contrast to the mitochondrial studies, that the soluble ATPase from *E. coli* is inhibited by the water soluble bathophenanthroline sulfonate at concentrations similar to bathophenanthroline (table 5) which indicates that detachment of the ATPase from the membrane may expose a chelator sensitive site.

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