Inhibition by Mercurial Reagents and Role of SH Groups of the Adenosine Triphosphatase from *Escherichia coli* 414 Membranes

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Mercurials selectively inhibited *Escherichia coli* 414 ATPase (EC 3.6.1.3). Inhibition of the soluble ATPase (BF₁) was greater than for the membrane-bound enzyme. The titration of 4 SH groups per mol BF₁ with 0.05 mM-*p*-chloromercuri[¹⁴C]benzoate showed a good dose-response curve for the inhibition of basal ATPase but not for the trypsin-stimulated activity. Accessible SH groups did not seem to be related to the active site of the enzyme. Mercurials appeared to affect *E. coli* ATPase by inducing a molecular change in the holo-enzyme, followed by dissociation. One to two SH groups with different degrees of accessibility were located in the α and γ subunits of ATPase (BF₁) but only one was located in the β subunit, irrespective of the concentration of *p*-chloromercuribenzoic acid, suggesting a structural role for SH groups in BF₁.

INTRODUCTION

Of the specific components of the energy-transducing machinery, the ATPases or coupling factors F₁ (EC 3.6.1.3) have been most actively investigated owing to the ease with which they may be solubilized, so allowing their purification and molecular characterization (Penefsky, 1974; Abrams & Smith, 1974; Pedersen, 1975; Nelson, 1976). The F₁ factors from mitochondria and chloroplasts (Penefsky, 1974; Nelson, 1976) have been thoroughly studied but studies on bacterial ATPases or BF₁ factors have been less comprehensive. Studies carried out in our laboratory on the water-soluble ATPase portion (BF₁ factor) of the ATPase-ATP synthetase complex from two strains of *Micrococcus lysodeikticus* (Andreu *et al.*, 1973; Carreira *et al.*, 1976; Carreira *et al.*, 1977) and one strain of *Escherichia coli* (Azocar & Muñoz, 1977) have demonstrated differences between bacterial energy-transducing ATPases (Andreu *et al.*, 1973; Carreira *et al.*, 1977; Azocar & Muñoz, 1977). A similar conclusion on possible differences in the molecular structure of mitochondrial and chloroplast F₁ was reached in recent studies on the analysis of the cross-linked products from both these ATPases (Baird & Hammes, 1976, 1977). At present, a comparative biochemical study of F₁ factors from different origins appears to be worthwhile for understanding the structure-function relationships of these energy-transducing proteins.

One of the less explored areas in the study of BF₁ factors is the possible involvement of SH groups in their structure and function. Both mitochondrial and chloroplast ATPases contain 12 half-cystines per molecule – eight SH and four in two disulphide bonds (Senior, 1975; Farron & Racker, 1970). On the other hand, the half-cystine content of bacterial ATPases is seldom cited in the literature and, when available, appears to be much lower than that of mitochondrial and chloroplast ATPases (Andreu *et al.*, 1973; Monteil *et al.*, 1975). Apart from some indirect observations that *p*-chloromercuribenzoate or other SH

reagents inhibited the membrane ATPase from *E. coli* strains and *Streptococcus faecalis* (Evans, 1969; Kobayashi & Anraku, 1972; Gunther *et al.*, 1974; Nelson *et al.*, 1974; Ahlers *et al.*, 1975), the effect of such inhibitors on BF₁ has not been studied. In a recent report, we demonstrated the role of thiol groups and disulphide bridges in maintaining the structural integrity of the cytoplasmic membrane from *E. coli* K12 (Azocar & Muñoz, 1976). These results prompted us to investigate the significance of thiol group modification on the properties of the membrane ATPase from *E. coli* 414. The present report describes the selective effect of mercurial reagents on the properties of this ATPase in its membrane-bound and soluble states. This effect has been examined on the unmasked ATPase activity, referred to as basal activity, as well on the latent activity which may be elicited by trypsin digestion of the natural inhibitor of the ATPase (trypsin-stimulated activity). Inhibition of ATPase is probably the consequence of a dissociation of the holoenzyme induced by the action of the reagents on accessible SH groups. This effect has not hitherto been reported and suggests a role for SH groups in the structural domains of the protein, for example, at the inter-subunit regions. Under the same experimental conditions, purified ATPase BF₁ from *M. lysodeikticus* was not affected by the mercurial reagents.

METHODS

Organisms. *Escherichia coli* 414 (Hfr, *thr*), a derivative of strain K12, which was a gift from Dr J. Puig (Universidad de los Andes, Mérida, Venezuela), and *Micrococcus lysodeikticus* strain PWYE or strain A (Andreu *et al.*, 1973; Carreira *et al.*, 1976) were grown as previously described (Corao *et al.*, 1974; Carreira & Muñoz, 1975; Carreira *et al.*, 1976).

Membrane isolation; solubilization and purification of ATPase. Membrane 'ghosts' were isolated by the sequential treatment of exponentially growing *E. coli* with EDTA and lysozyme as described previously (Corao *et al.*, 1974; Carreira & Muñoz, 1975). Membranes were kept as pellets for periods of not longer than 1 week and were used as a source of membrane-bound ATPase after resuspending them in 30 mM-Tris/HCl, pH 7.5, containing 1 mM-MgCl₂ at about 1 mg protein ml⁻¹. The ATPase of *E. coli* was solubilized by suspending the Mg²⁺-free membrane pellets in 50 mM-Tris/HCl, pH 9.0, containing 3 mM-EDTA for 15 min at the same protein concentration as above; the enzyme remained in the supernatant after centrifugation at 27000 g (Carreira *et al.*, 1973; Carreira & Muñoz, 1975). Soluble ATPase was obtained by filtering the supernatant through an Amicon Diaflo PM-10 membrane (1 to 1.5 kgf cm⁻²) to eliminate EDTA (Azocar & Muñoz, 1977). The ATPase was purified by molecular sieving as described elsewhere (Azocar & Muñoz, 1977).

Membranes and ATPase from *M. lysodeikticus* strain A were obtained as previously described (Muñoz *et al.*, 1969; Andreu *et al.*, 1973; Andreu & Muñoz, 1975).

Assay of ATPase activity. Basal and trypsin-stimulated activities of *E. coli* preparations were measured using 8 mM-ATP in the presence of 4 mM-MgCl₂ essentially as described by Carreira & Muñoz (1975). This substrate concentration was about sevenfold higher than that required for half-saturation of the enzyme. Activity of *M. lysodeikticus* ATPase was measured using 8 mM-ATP/Ca²⁺ as substrate and the conditions previously described (Muñoz *et al.*, 1969).

The effect of thiol reagents on ATPase activity was tested by preincubating ATPase preparations with any of the reagents at final concentrations ranging from 10 µM to 3 mM. Occasionally, an excess of dithiothreitol was added at the end of the incubation with the thiol reagent to reverse its effect.

Determination of SH groups in *E. coli* ATPase preparations. Thiol groups were determined either with 1 mM-5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Azocar & Muñoz (1976), or by measuring the retention on nitrocellulose filters of the complex formed between *p*-chloromercuri[¹⁴C]benzoic acid (CMB) and protein SH groups, following the procedure described by Krakov & Goolsby (1971).

Polyacrylamide gel electrophoresis and identification of bands labelled with *p*-chloromercuri[¹⁴C]benzoate. Gel electrophoresis was carried out at pH 8.5 ± 0.1 using the Tris/glycine system or at pH 7.5 using the Tris/acetate system as described by Azocar & Muñoz (1977). Gels (11 × 0.5 cm) containing 7 % (w/v) acrylamide were used in both cases. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out as described above but with the addition of 0.1 % (w/v) SDS to both gels, and buffer and samples were pretreated with 8 mg SDS (mg protein)⁻¹.

Proteins were stained with Coomassie 250 as described by Andreu *et al.* (1973). The distribution of radioactivity in protein was followed by laterally slicing the gels into 0.8 mm discs with a Mickle Gel Slicer and treating the slices as follows. To each slice, 0.3 ml 0.1 % (w/v) SDS was added; the slices were then frozen and thawed three times, shaken overnight at 30 °C and suspended in Bray's solution (Bray, 1960). Alternatively,

the slices were directly soaked in 3 ml of a scintillation solution containing 0.5 % (w/v) 2-(4'-*tert*-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole (butyl-PBD) and 33 % (v/v) Triton X-100 in toluene and counted after 1 h shaking. The efficiency of counting ^{14}C was 40 % by both methods, though the latter method was less time-consuming.

RESULTS

Quantitative distribution of SH groups in E. coli membranes and soluble ATPase

The soluble ATPase fraction from *E. coli* membranes, representing 10% of total membrane protein, contained 0.9 mol accessible SH groups (50 kg protein) $^{-1}$, as measured by titration with DTNB and, following denaturation with SDS, 1.8 mol total SH (50 kg protein) $^{-1}$. The whole membranes contained 0.8 mol accessible SH (50 kg protein) $^{-1}$ and 3.4 mol total SH (50 kg protein) $^{-1}$. Therefore, the soluble ATPase was less enriched in SH than the whole membranes, though its thiol groups were more accessible to titration with DTNB without denaturation of the protein. Thus, the SH groups measured with SDS present in the soluble ATPase preparation accounted for only 5% of the total SH of membranes while those titrated without SDS represented 11% of the membrane accessible SH groups.

Effect of thiol reagents on ATPase activity of E. coli membranes and soluble fraction

Thiol reagents inhibited *E. coli* ATPase (Table 1). The most effective inhibitor was CMB, followed by Hg^{2+} , DTNB, and the alkylating agents. Differences in sensitivity to these inhibitors were found between the membrane-bound and the soluble forms of ATPase, on one hand, and between the basal and trypsin-stimulated activities, on the other. The membrane-bound ATPase was only partially inhibited by CMB and the inhibition was greater for the trypsin-stimulated activity than for the basal activity. The level of inhibition reached with 10 μM -CMB was not increased by increasing the CMB concentration to 1 mM. However, for the soluble ATPase, the basal and trypsin-stimulated activities were inhibited to a similar extent by a given concentration of CMB and this inhibition increased from about 30% to 90% when the CMB concentration was increased from 0.1 mM to 0.3 mM.

The substrates, ATP and Mg^{2+} , protected the soluble and purified *E. coli* ATPase preparations from inhibition by CMB but they showed little protection for the membrane-bound enzyme (Table 2).

Table 1. *Effect of different thiol reagents on the activity of membrane-bound and soluble ATPase from E. coli 414*

The ATPase preparations (1 mg membrane protein ml $^{-1}$ or 200 μg soluble protein ml $^{-1}$ in 30 mM-Tris/HCl, pH 7.5) were incubated with different concentrations of the reagents listed below for 30 to 45 min at room temperature and subsequently analysed for ATPase activity.

Enzyme preparation	Reagent	Concn (mM)	Inhibition (%)	
			Basal ATPase	Trypsin- stimulated ATPase
Membrane-bound	CMB	0.01–1.00	20	48
	DTNB	0.25–1.00	0	0
	<i>N</i> -Ethylmaleimide	1.00	0	0
	Iodoacetamide	0.10–2.00	0	0
Soluble	CMB	0.01–0.10	24	35
	CMB	0.30–1.00	84	92
	Hg^{2+}	0.33	31	22
	DTNB	0.25–1.00	15 $\pm$ 5	21 $\pm$ 10
	<i>N</i> -Ethylmaleimide	0.50–3.00	16 $\pm$ 5	23 $\pm$ 6
	Iodoacetamide or iodoacetic acid	0.10–2.00	0–10	0–15

Table 2. *Effect of ATP and Mg²⁺ on the inhibition by p-chloromercuribenzoic acid of the membrane-bound and soluble ATPase from E. coli 414*

The ATPase preparations (1 mg membrane protein ml⁻¹ or 200 µg soluble protein ml⁻¹ in 30 mM-Tris/HCl, pH 7.5) were incubated with 1 mM-CMB alone or in the presence of 10 mM-Mg²⁺ or 10 mM-ATP for 45 min at room temperature and subsequently analysed for ATPase activity.

Enzyme preparation	Addition	Inhibition (%)	
		Basal ATPase	Trypsin-stimulated ATPase
Membrane-bound	None	20	42
	Mg ²⁺	12	48
	ATP	16	40
Soluble	None	84	92
	Mg ²⁺	22	56
	ATP	25	63
Purified	None	77	82
	Mg ²⁺	28	56
	ATP	18	40

Under identical experimental conditions, neither the membrane-bound ATPase nor the purified enzyme from *M. lysodeikticus* was inhibited by any of the SH reagents. Thus, these results show a clear difference between the ATPases of *M. lysodeikticus* and *E. coli* and suggest the existence of subclasses of accessible SH groups in *E. coli* ATPase.

Titration of SH groups in purified E. coli ATPase and inhibition of its activity

The possible existence of different classes of accessible SH groups was examined by attempting to correlate inhibition of the ATPase activity with titratable SH groups. The titration of 4 SH groups per molecule of enzyme was accompanied by inhibition of the basal activity of purified ATPase (Fig. 1). The correlation between inhibition and titration of SH groups was less good for the trypsin-stimulated activity. At 0.03 mM-CMB only 20% inhibition of the latter activity was observed while the inhibition increased to 40% at 0.05 mM-CMB though the number of SH groups titrated remained the same. At concentrations of CMB which produced about 80% inhibition of *E. coli* ATPase (see Tables 1 and 2) there was no correlation between the level of inhibition and the titration of 2 to 3 additional SH groups (Table 3). These observations confirmed the existence of subclasses of SH groups in *E. coli* ATPase, yet suggested that thiol groups were not involved in the active site of *E. coli* ATPase.

Effect of CMB on E. coli ATPase as studied by gel electrophoresis

In view of the last suggestion, the possibility that CMB may induce structural changes in *E. coli* ATPase was examined by gel electrophoresis. In both electrophoretic systems, samples treated with CMB showed a decrease in the level of ATPase holoenzyme, that is, in bands of relative mobility 0.10 in Tris/acetate buffer and relative mobility 0.24 in Tris/glycine buffer (Fig. 2). The effect was more evident when the electrophoresis was carried out at pH 8.5. Under these conditions the enzyme is very sensitive to any environmental alteration (Azocar & Muñoz, 1977). The addition of dithiothreitol to the incubation mixtures and during gel electrophoresis reversed the molecular changes induced by CMB. Other mercurials such as Hg²⁺ or mersalyc acid induced a similar structural change, possibly a dissociation, in *E. coli* ATPase.

Radioactivity was detected in three bands following similar experiments using [¹⁴C]CMB (Fig. 2c). One band corresponded to ATPase holoenzyme and the other two bands (relative

Fig. 1. Titration of SH groups in purified *E. coli* ATPase and inhibition of its activity by *p*-chloromercuribenzoic acid. Samples of purified ATPase (150 μg) in 500 μl 30 mM-Tris/HCl, pH 7.5, were incubated at 20 °C with various concentrations of [¹⁴C]CMB (specific activity 1.05×10^6 c.p.m. μmol⁻¹). After 45 min, 100 μl portions (containing about 10 μg protein) were removed for assay of basal and trypsin-stimulated ATPase activity. The residual volume (300 μl) was filtered through nitrocellulose filters and the complexes formed between [¹⁴C]CMB and protein SH groups were measured (Krakow & Goolsby, 1971). ○, mol SH titrated; ●, inhibition of basal ATPase; □, inhibition of trypsin-stimulated ATPase.

Table 3. Inhibition of purified ATPase from *E. coli* 414 by *p*-chloromercuribenzoic acid and correlation with titration of its SH groups

Purified *E. coli* ATPase (100 μg) was incubated with different concentrations of [¹⁴C]CMB (specific activity 1.05×10^6 c.p.m. μmol⁻¹) in 500 μl 30 mM-Tris/HCl, pH 7.5, for 45 min at 20 °C. After incubation, portions (containing about 10 μg protein) were removed for assay of ATPase activity and the remainder of the mixture was filtered. The filters were washed with 1 ml 100 mM-Tris/HCl, pH 7.5 and dried, and radioactivity was counted either directly or in the presence of 3 ml Bray's solution (Bray, 1960).

CMB concn (mM)	Inhibition (%)		SH groups [mol (mol ATPase) ⁻¹]
	Basal ATPase	Trypsin- stimulated ATPase	
0.03	38	20	4
0.05	38	40	4
0.30	75	80	4.5
1.00	77	82	6.7

mobilities 0.84 and 0.86) corresponded to the major subunits. These components were identified by eluting the appropriate bands and subjecting them to SDS-polyacrylamide gel electrophoresis.

Location of SH groups in the subunits of E. coli ATPase

Exposure of *E. coli* ATPase to two different [¹⁴C]CMB concentrations yielded qualitatively similar radioactivity profiles on SDS-polyacrylamide gel electrophoresis (Fig. 3). Radioactivity was incorporated into the α, β and γ subunits, but quantitative differences between subunits were observed. The α and γ subunits were more heavily labelled after exposure to 1 mM-[¹⁴C]CMB. At 50 μM-CMB, the α, β and γ subunits contained about 1 mol labelled SH per mol polypeptide, while at 1 mM-CMB, 2 mol of SH per mol of α and γ subunits were labelled. These results are therefore consistent with those reported in Table 3.

Fig. 2. Polyacrylamide gel electrophoresis of purified ATPase from *E. coli* 414 showing the effect of CMB. Purified ATPase (15 μ g) in 100 μ l 30 mM-Tris/HCl, pH 7.5, was incubated (a, b) with (---) or without (—) 1 mM unlabelled CMB or (c) with [14 C]CMB (specific activity 2×10^5 c.p.m. μ mol $^{-1}$). After 45 min at 20 $^{\circ}$ C, mixtures were electrophoresed in (a) Tris/acetate, pH 7.5, or (b, c) Tris/glycine, pH 8.5. Migration was towards the anode (+).

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified *E. coli* ATPase labelled with [14 C]CMB. Samples of purified ATPase (50 μ g) were incubated with either (a) 0.05 mM- [14 C]CMB or (b) 1 mM- [14 C]CMB (specific activity 1.05×10^6 or 2×10^5 c.p.m. μ mol $^{-1}$, respectively) for 45 min at 20 $^{\circ}$ C. The labelled protein was separated from excess reagent by Sephadex G-25 gel chromatography on a column (0.9 $\times$ 25 cm) and then dissociated with 1% (w/v) SDS. The dissociated samples were electrophoresed in Tris/glycine, pH 8.5, containing 0.1% (w/v) SDS and the gels were treated as described in Methods. Bars indicate the positions of the α , β and γ subunits.

DISCUSSION

The mechanism by which mercurial reagents inhibited *E. coli* ATPase deserves comment. On one hand, the concentration-response curve of basal ATPase activity was correlated with the modification of 4 mol SH per mol enzyme suggesting that these SH groups were involved in the binding or hydrolytic site of the ATPase. However, the lack of correlation between the titration of these 4 SH groups and the inhibition of the trypsin-stimulated activity argued against this suggestion. The latter observation was supported by labelling experiments with [^{14}C]CMB which yielded 50% of the label in dissociation products of the ATPase (see Fig. 2).

Taken together, the results suggest that free, accessible SH groups are not directly involved in the binding or catalytic site of *E. coli* ATPase. The evidence points to a different mechanism of inhibition. It appears likely that inhibition of *E. coli* ATPase by mercurials results from profound structural change and concomitant dissociation of the holoenzyme. This effect is reminiscent of that observed on depletion of Mg^{2+} from *E. coli* $\kappa 12$ ATPase by EDTA (Azocar & Muñoz, 1977). It is therefore tempting to suggest that mercurials affect *E. coli* ATPase by their ability to chelate and/or compete with divalent cations. This may account for the selective action of these reagents on *E. coli* ATPase. Alternatively, the selective effect of mercurials may be related to their relative greater hydrophobicity compared with other SH group reagents since the effectiveness of these reagents in inhibiting *E. coli* ATPase correlated with their degree of hydrophobicity (see Table 1). Mersalyc acid was also a potent inhibitor of *E. coli* ATPase. Irrespective of their exact mode of action, the effect of mercurials on *E. coli* ATPase was on thiol groups, as confirmed by reversal of their effect by dithiothreitol.

These studies have provided evidence for the existence of subclasses of SH groups in *E. coli* ATPase, including the presence of buried SH groups. One subclass of accessible SH groups, present in approximately equimolar amounts in the α , β and γ subunits, was modified by low concentrations of CMB. Another class, only present in α and γ subunits, required higher concentrations of CMB before reaction occurred.

The studies reported here provide new insight into the idea that energy-transducing proteins display differences in molecular organization. Under the same experimental conditions, *M. lysodeikticus* and *E. coli* ATPases behaved differently in their sensitivity to mercurials. We do not know whether the inhibition by mercurials reported here is a general property of the ATPases from all *E. coli* strains or is strain-specific.

Our observations on *E. coli* ATPase agree with those of Pedersen (1976), who found a selective inhibitory effect of mercurials on the bicarbonate stimulated-ATPase activity of mitochondrial F_1 and related it to a conformational change of the mitochondrial enzyme rather than to a direct involvement of SH groups at the catalytic site.

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