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CROSSLINKING STUDIES ON THE CA^{2+}, MG^{2+}-ACTIVATED ATPASE OF ESCHERICHIA COLI

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Crosslinking of membrane proteins of Escherichia coli with dithiobis (succinimidyl propionate) (DSP) resulted in loss of several enzyme activities including the Ca^{2+}, Mg^{2+}-activated ATPase. This enzyme was crosslinked by DSP to the membrane and was not released by dialysis at low ionic strength in the absence of dithiothreitol which could cleave the crosslinking group. DSP inactivated both phosphohydrolase and coupling activities of the solubilized ATPase. Loss of hydrolytic activity could be correlated with the extent of reaction of the α and/or β subunits of the enzyme. The loss of coupling activity appeared to be associated with modification of the γ and/or δ subunits.

INTRODUCTION

The energy-transducing Ca^{2+}, Mg^{2+}-activated ATPase of E. coli solubilized from the cell membrane contains five types of subunit polypeptides (α–ϵ) having a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (1). In a previous study of the arrangement of these polypeptides in the ATPase molecule, we showed that the crosslinking agent, dithiobis (succinimidyl propionate) (DSP) (Fig. 1), linked α to β subunits, but not α to α or β to β subunits (1). A crosslinked product containing α, β, and γ subunits was also detected. On the basis of this data we suggested that the three α and the three β subunits in the enzyme molecule were arranged alternately as a planar hexagon about the central γ subunit, in agreement with the appearance of the enzyme as seen in electron micrographs.

In the previous study we did not examine the effects of the crosslinking agent on the enzymic properties of the ATPase. In this paper we report on the effect of DSP on the activities of both soluble and membrane-bound ATPases. Furthermore, we show that the ATPase can be crosslinked to components in the membrane so that it cannot be released by procedures normally used to solubilize the enzyme.

METHODS

E. coli NRC482 was grown at 37°C to the late exponential phase with vigorous aeration on a minimal salts medium containing 22 mM glucose and 12 μM ferric citrate.

Washed membrane particles were prepared as previously described and stripped of ATPase by dialysis overnight against 1 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol (2). The dialyzed particle suspension was

centrifuged at 120,000 X g for 2 hr to yield a pellet of "stripped" membrane particles. ATPase was purified to homogeneity from the supernatant by chromatography on DEAE-cellulose, followed by sucrose gradient centrifugation. The procedure followed the method used previously (2), except that chromatography on Sepharose 6B was not used, and the sucrose gradient was prepared in triethanolamine instead of Tris buffer and dithiothreitol was omitted.

Ca^{2+}-activated ATPase, respiration- and ATP-driven energy-dependent transhydrogenase activities were measured at 37°C, as previously described (3), except that the final concentration of sucrose in the transhydrogenase assays was 0.133 M. Energy-independent transhydrogenase and NADH oxidase activities were measured by the methods of Singh and Bragg (4). Analytical gel electrophoresis was performed either on 5% polyacrylamide gels prepared and run in 0.05 M Tris-glycine buffer, pH 8.7, or on 7.5% gels containing 0.1% sodium dodecyl sulfate (SDS) as previously described (2). For the latter system, samples were depolymerized by heating at 100°C for 3 min with 4 M urea containing 1% SDS. Protein bands were stained with Coomassie blue and the gels scanned at 500 nm with a Gilford model 240 spectrophotometer equipped with a linear transporter.

RESULTS AND DISCUSSION

Effect of DSP on Membrane-Bound Enzyme Activities

The effect of DSP on the activities of some selected membrane enzymes is shown in Fig. 2. The enzymes were not equally sensitive to inhibition by DSP. Thus, energy-independent transhydrogenase activity (and respiration- and ATP-driven transhydrogenase activities – data not shown) was 50% inactivated at 0.025 μmoles DSP/mg membrane protein compared to 0.1–0.15 μmoles DSP/mg portein required for inhibition of the Ca^{2+}, Mg^{2+}-activated ATPase and the NADH oxidase. Inhibition of these enzymes was at least partially reversed by incubating the treated membranes with 60 mM dithiothreitol (DTT). DTT is able to cleave the disulfide bridge in the crosslinked products, but is unable to remove the β-mercaptopropionyl substituents from amino groups of reacted proteins. Thus, the partial reversal by DTT of inhibition is indicative that at least some of the inhibition was due to crosslinking.

Crosslinking of the ATPase to the membrane by DSP was suggested by following results. Membrane particles in 0.2 M triethanolamine buffer, pH 8.5, containing 10% glycerol were treated with DSP to give a final concentration of 0.5 μmoles DSP/mg protein. After 5 min excess DSP was destroyed by the addition of 4M Tris-HCl buffer, pH 8.5, and the treated particles were isolated by ultracentrifugation. They were suspended in dialysis buffer and then dialyzed against several changes of 1 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 10% glycerol. This procedure releases almost all of the ATPase from untreated membranes (2). The dialyzed particles were sedimented by ultracentrifugation and the supernatant was examined for the presence of the characteristic α and β subunits of the ATPase on SDS gels following disaggregation in 1% SDS-4 M urea-1% β-mercaptoethanol (2). As shown in Fig. 3, no subunits of the ATPase were detected in the material released by dialysis. The bands of the α and β subunits of the enzyme are the most strongly staining bands observed on gels of the corresponding fraction from un-

treated membranes. The pellet was resuspended in dialysis buffer and dialyzed overnight against the same buffer supplemented with 1 mM DTT. The supernatant fraction was isolated as before and examined on SDS gels (Fig. 3). The α and β subunits of the ATPase were now apparent. These results suggest that DSP can crosslink the ATPase molecule to the membrane, preventing its release by the dialysis procedure until the disulfide band in the crosslinker has been cleaved by reduction with DTT.

The ability to crosslink the ATPase to the membrane is potentially a useful tool for the identification of those proteins in the membrane with which the ATPase can interact. This relationship is presently being examined.

Effect of DSP on the Soluble Ca^{2+}, Mg^{2+}-Activated ATPase

The effect of DSP on the ATPase was examined in greater detail using a homogeneous, soluble preparation which had been released from the membrane. As shown in Fig. 4, DSP inhibited the hydrolytic activity of the purified ATPase and the effect could be partially reversed by subsequent treatment with DTT, indicating that at least part of the loss of activity was due to crosslinking within the ATPase molecule. At 1 μmole DSP/mg protein, there was no crosslinking between different ATPase molecules as shown by polyacrylamide gel electrophoresis and sucrose gradient centrifugation. Half-maximal inhibition was obtained at 0.6 μmoles DSP/mg protein compared to 0.1 μmoles/mg protein for the membrane-bound enzyme.

A characteristic property of the purified ATPase is its ability to restore both respiration- and ATP-driven transhydrogenase activities to ATPase-stripped membrane particles (2). This coupling activity of the purified ATPase was half-inactivated at about 0.2 μmoles DSP/mg protein. In all of the reconstitution experiments, excess DSP was destroyed by reaction with Tris. Although the reaction products were not removed, the maximum amount which could be present in the preparation of DSP-treated ATPase added to the particles for reconstitution would be 6 nmoles/mg particle protein at the concentration of DSP required for half-inactivation of the coupling activity. This amount of DSP or of its reaction products would not cause significant inhibition of transhydrogenase activities (Fig. 2).

An attempt was made to correlate the modification by DSP of the subunits of the ATPase with the loss of hydrolytic and coupling activities by examining the extent of disappearance of the subunit bands, as observed on SDS gels of the ATPase following treatment with various levels of DSP. As shown in Fig. 4, the bands of the γ and δ subunits disappeared more readily from the gels than those of the α and β subunits. As we have shown elsewhere (1), this apparent loss of subunits is due to crosslinking into products of higher molecular weight. The loss of ATPase activity in the presence of DSP correlates most closely with the extent of crosslinking of the α and β subunits. Loss of coupling activity seemed to be related to the extent of reaction of the γ and δ subunits of the ATPase.

The effect of DSP on the binding of the ATPase to the stripped membrane was explored with enzyme which had been treated with a concentration of DSP sufficient almost completely to inhibit coupling activity but having substantial (42%) hydrolytic activity remaining. As shown in Table I, in contrast to the untreated ATPase, the DSP-treated enzyme could not significantly restore respiration- or ATP-driven transhydrogenase

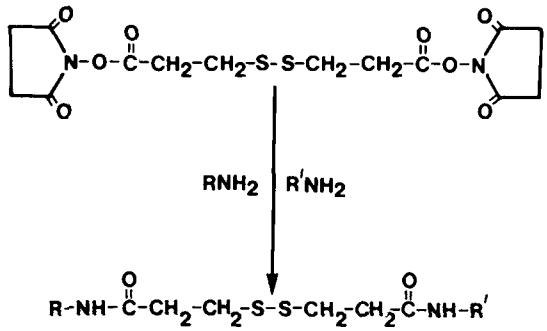

Fig. 1. Crosslinking of protein groups RNH_2 and $R'NH_2$ with dithiobis (succinimidyl propionate) (DSP).

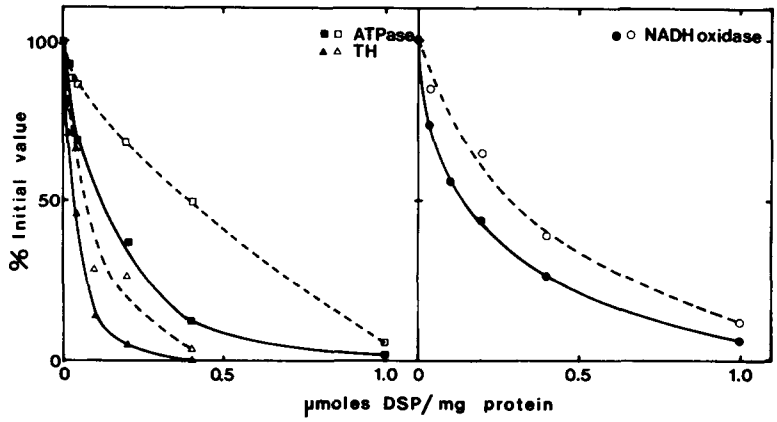

Fig. 2. Loss of ATPase, energy-independent transhydrogenase (TH), and NADH oxidase activities of membrane particles on treatment with DSP. Membrane particles (1.25 mg protein) in 0.1 ml 0.2 M triethanolamine-HCl buffer, pH 8.5, containing 10% glycerol were treated with the indicated concentrations of DSP for 2 min at 22°C. The reaction was terminated by the addition of 0.025 ml 4 M Tris-HCl buffer, pH 8.5, with or without 0.3 M DTT. After a further 2 min, samples were removed for assay. The specific activities (nmoles/min/mg protein) of the ATPase, energy-independent transhydrogenase, and NADH oxidase in the untreated particles were 740, 329, and 550 in the absence of DTT, and 800, 323, and 516 in the presence of DTT, respectively. Closed points, -DTT; open points, +DDT.

activities to ATPase-stripped membrane particles. Moreover, the treated enzyme could not prevent the reaction of the untreated enzyme with the particles to reconstitute transhydrogenase activities, even when it was incubated with the particles prior to the addition of the untreated enzyme. It is not clear if the binding of the DSP-treated ATPase to the membrane is impaired. Addition of untreated or DSP-treated ATPase to stripped particles resulted in the binding of 26% and 15%, respectively, of the added ATPase units to the membrane (Table II). The first value probably represents complete saturation of

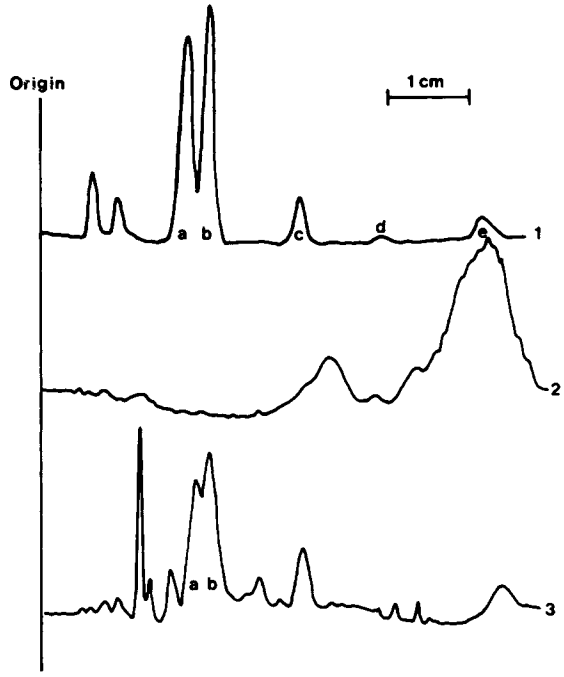

Fig. 3. Crosslinking of ATPase to membrane by DSP. Membrane particles (88 mg protein) in 9 ml 0.2 M triethanolamine buffer, pH 8.5, containing 10% glycerol were stirred with DSP (45 μmoles in 0.09 ml dimethylsulfoxide) for 5 min at 20°C. The reaction was terminated by the addition of 9 ml 4 M Tris-HCl buffer, pH 8.5. The treated membrane particles sedimented by centrifugation at 120,000 × g for 2 hr were suspended in 5 ml 1 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 10% glycerol ("dialysis buffer") and dialyzed overnight at 4°C against 500 ml of the same buffer. Centrifugation of the dialyzed material at 120,000 × g for 2 hr yielded a pellet and a supernatant fraction ("supernatant A"). The pellet was suspended in 5 ml dialysis buffer containing 1 mM DTT and dialyzed overnight at 4°C against 500 ml of this buffer. "Supernatant B" was obtained by centrifugation of the dialyzed material as above. Samples of purified ATPase (curve 1), supernatant A (curve 2), and supernatant B (curve 3) were examined by electrophoresis on 7.5% polyacrylamide gels containing 0.1% SDS following disaggregation in 1% SDS-4 M urea-1% β-mercaptoethanol for 3 min at 100°C. Subunits α-- ε of the ATPase are indicated by a–e.

available binding sites for the ATPase on the stripped membrane, since the addition of both DSP-treated and untreated ATPase did not result in further binding of ATPase units above this value. However, the ATPase bound to the membrane from the treated preparation may represent DSP-modified ATPase, but could be native ATPase which had escaped reaction with DSP. The former alternative seems more probable since the binding of these ATPase units did not result in any reconstitution of transhydrogenase activity. If this suggestion is correct, then the untreated ATPase must be able to displace the DSP-treated ATPase from the binding sites to account for the full reconstitution of transhydrogenase activities when both treated and untreated enzymes are present.

The apparent correlation between the extent of reaction of the α and β subunits and the loss of ATPase activity is in agreement with the results of Nelson et al. (5) which

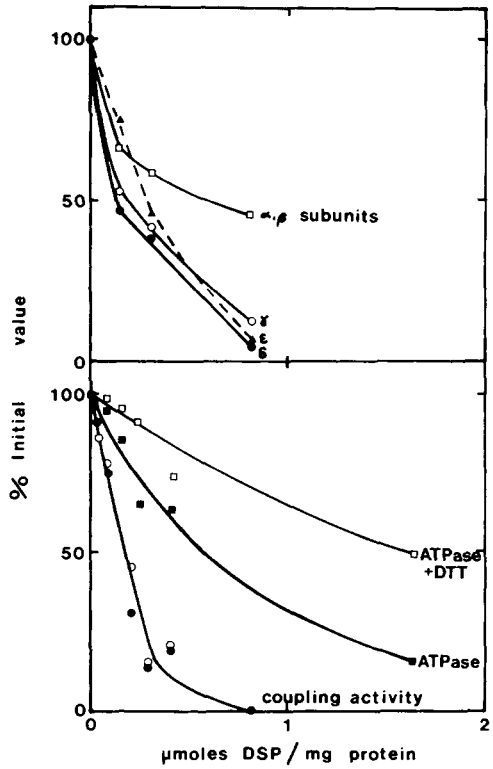

Fig. 4. Loss of ATPase activity, coupling factor activity for energy-dependent transhydrogenase, and subunits, on treatment of the ATPase with DSP. The ATPase (0.06 mg protein) in 0.05 ml 0.2 M triethanolamine-HCl buffer, pH 8.5, containing 11% sucrose, 5% glycerol, and 0.25 mM EDTA, was treated with the indicated concentration of DSP for 2 min at 22°C. The reaction was terminated by the addition of 5 μl 4 M Tris-HCl buffer, pH 7.8. Samples of the treated enzyme were analyzed for enzyme activities or were electrophoresed in SDS on 7.5% polyacrylamide gels. The concentration of the subunits was determined following staining with Coomassie blue. Coupling factor activity was measured as respiration-driven (open points) and ATP-driven (closed points) transhydrogenase activities of ATPase-stripped membrane particles (0.48 mg protein) in the presence of DSP-treated ATPase (61 μg protein). The initial activities with the untreated enzyme were' ATPase, 27 μmoles/min/mg protein; respiration-driven transhydrogenase, 33 nmoles/min/mg protein; ATP-driven transhydrogenase, 59 nmoles/min/mg protein.

showed that the phosphohydrolase activity of the ATPase was associated with the α and/or β subunits. It has been shown that removal of the δ subunit of the ATPase results in loss of coupling activity (6) and in binding to the membrane (7). Thus, the inhibitory effects of DSP on coupling activity, and possibly on binding, could be explained by the observed modification of the δ subunit of the enzyme.

ACKNOWLEDGMENTS

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TABLE I. Coupling Factor Activity of Normal and DSP-Treated ATPase for Energy-Dependent Transhydrogenase Activities of ATPase-Stripped Membrane Particles[1]

Incubation mixture	Transhydrogenase activity[2]	
	Respiration driven	ATP-driven
Particles	0	0
Particles + ATPase	51	91
Particles + DSP-ATPase	8	11
Particles + ATPase, then + DSP-ATPase	53	90
Particles + DSP-ATPase, then + ATPase	51	85
Particles + DSP-ATPase,[3] then + ATPase	50	74

[1] Stripped membrane particles (0.42 mg protein) in 1 ml 50 mM Tris-H_2SO_4 buffer, pH 7.8, containing 10 mM $MgCl_2$, 0.16 M sucrose and 0.1% bovine serum albumin, were preincubated at 37°C for 4 min, and then for a further 1 min, with normal and/or DSP-treated (0.25 μmoles DSP/mg ATPase protein. See legend to Fig. 4 for treatment method) ATPase (0.024 mg protein). The mixture was then assayed for respiration- and ATP-driven transhydrogenase activities.

[2] nmoles $NADP^+$ reduced/min/mg particle protein.

[3] 0.048 mg DSP-ATPase protein.

TABLE II. Binding of Normal and DSP-Treated ATPase to ATPase-Stripped Membrane Particles[1]

Incubation mixture	Time of incubation (min)	ATPase[2]			
		Pellet	Supernatant	Pellet[3]	Supernatant[3]
Particles	4	0.42	0.45	0	0
ATPase	4	0	5.97	0	5.97
DSP-ATPase	4	0	2.52	0	2.52
Particles + ATPase	4	1.94	4.28	1.52	3.83
Particles + DSP-ATPase	4	0.81	2.27	0.39	1.82
Particles + ATPase,	2				
then + DSP-ATPase	2	2.00	6.60	1.58	6.15
Particles + DSP-ATPase	2				
then + ATPase	2	1.87	5.72	1.45	5.27

[1] Normal or DSP-treated (0.25 μmoles DSP/mg ATPase protein. See legend for Fig. 4 for treatment method) ATPase (0.096 mg protein) was incubated at 37°C with stripped membrane particles (1.68 mg protein) in 4.5 ml 0.05 M Tris-H_2SO_4, pH 7.8, containing 0.01 M $MgCl_2$, 0.16 M sucrose, and 1 mg/ml bovine serum albumin. The mixture was centrifuged at 224,000 × g for 2 hr and the distribution of ATPase measured in the resulting pellet and supernatant.

[2] μmoles/min.

[3] Values corrected for ATPase contained in or released from particles.

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