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THE MEMBRANE ATPase OF *ESCHERICHIA COLI*

II. RELEASE INTO SOLUTION, ALLOTOPIC PROPERTIES AND RECONSTITUTION OF MEMBRANE-BOUND ATPase

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SUMMARY

Membrane-bound ATPase of *Escherichia coli* was released in a soluble form by decreasing the Mg^{2+} concentration to 0.05 mM. The particulate fraction left behind was depleted by more than 90% from its initial ATPase activity.

Soluble ATPase exhibits a number of different properties as compared with membrane-bound ATPase. These are a 2-fold increased K_m toward ATP, a shift of 1–1.5 pH units in the pH-dependence curve, a greatly increased resistance to inhibition by *N,N'*-dicyclohexylcarbodiimide (DCCD) and a stimulation by Dio 9 instead of an inhibition.

Upon mixing the soluble fraction and the depleted membrane fraction, the initial properties of native membrane-bound ATPase reappear. This reconstitution requires Mg^{2+} and results in the physical binding of the activity to sedimentable material.

Soluble ATPase and depleted membrane can be titrated against each other until an equivalence point is reached, beyond which the component in excess keeps its previous characteristics.

During the release procedure, DCCD remains associated with the particulate fraction with conservation of the ATPase-binding sites.

Such DCCD-treated depleted membranes behave as a specific inhibitor of soluble ATPase.

INTRODUCTION

The membrane-bound ATPase activity of *Escherichia coli* described in a previous article[1] appeared as essentially homogeneous with respect to substrate concentration dependence and sensitivity to inhibitors, among which *N,N'*-dicyclohexylcarbodiimide (DCCD), azide and Dio 9 are typical inhibitors of oxidative phosphorylation. These facts and the finding of ATPase-deficient mutants[2–4], unable to grow on strictly aerobic substrates such as succinate and/or lactate, but

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

utilizing fermentable sugars, strongly suggest that this activity is due to the terminal enzyme of the ATP-synthesizing system, and is therefore homologous to the mitochondrial ATPase which is called coupling factor F1[5].

In the present article we describe the release of ATPase from membrane particles, the changes in kinetic behaviour and in sensitivity to various inhibitors which accompany this release, and the reconstitution of membrane-bound activity with an essentially complete return to the native properties of membrane ATPase. The kinetic and pharmacodynamic properties which undergo reversible changes when an enzyme is moved from its membrane location into aqueous solution and back were called allotopic properties[6].

MATERIAL AND METHODS

Bacteria and membrane preparations have been described previously[1]. In brief, *E. coli* K12 strain 3300 was harvested in the exponential growth phase, disrupted in a Ribi fractionator under 23 000 lb/inch^2 pressure in 1 mM Tris–HCl buffer (pH 7.6) containing 1 mM $MgCl_2$. Particles sedimenting between 40 000 × *g* (30 min) and 165 000 × *g* (120 min) were resuspended in the same buffer at a concentration of approximately 50 mg protein per ml, and were conserved frozen (for utilization after thawing) at an appropriate dilution.

Preparation of crude solubilized ATPase and of depleted membrane particles

The membrane preparation was diluted in 20 vol. of 1 mM Tris–HCl (pH 7.6) at room temperature. This brought the concentration of Mg^{2+} to approximately 0.05 mM.

The diluted membranes were centrifuged for 120 min at 165 000 × *g*. The supernatant contained the solubilized ATPase and was either used fresh, or was precipitated with $(NH_4)_2SO_4$ added to 60% saturation and kept in the refrigerator (4 °C). The pellet was resuspended in the original volume of the original 1 mM Tris–HCl buffer (pH 7.6) containing 1 mM $MgCl_2$; it contained the depleted membrane particles which were used fresh or kept frozen as in the case of the native membranes.

ATPase assays were performed as described previously[1] in 40 mM triethanolamine–HCl buffer at pH 7.5 with 3.3 mM ATP and 1.66 mM Mg^{2+}. After 4 min of incubation at 28 °C the inorganic phosphate was measured by the method of Martin and Doty[7] unless stated otherwise.

RESULTS

Release of ATPase

Typical results of a standard experiment of release are summarized in Table I.

Fig. 1 summarizes the results obtained where the standard Tris buffer in the depletion experiment was replaced by a variety of electrolyte solutions. Fig. 1a shows that the ATPase activity which disappears from the pellet is proportional to the ATPase activity which appears in the supernatant, although the latter is only about 40% of the former (at pH 7.5). Fig. 1b shows the correlation between the extent of release and the Mg^{2+} concentration in the diluted membrane sus-

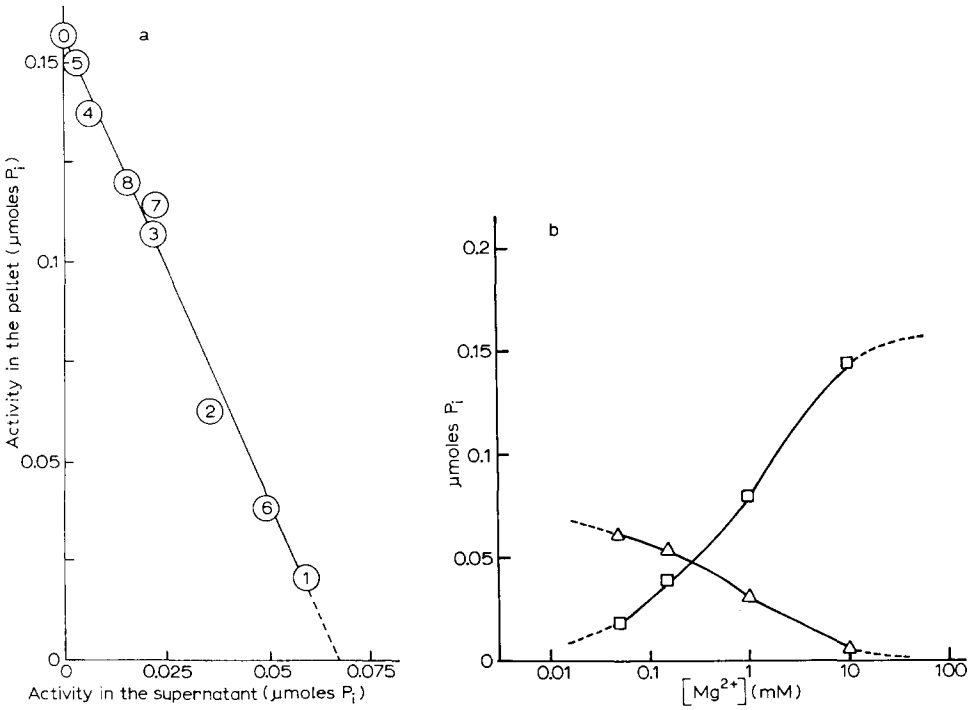

Fig. 1. Effect of Mg^{2+} and ionic strength on the release of ATPase from membranes. Each sample of initial membranes prepared as described in Material and Methods was diluted in 20 vol. of appropriate buffer at pH 7.5 and centrifuged for 120 min at 165 000 × *g*. The pellet was resuspended in the original volume of 1 mM Tris–HCl (pH 7.5). ATPase activity was measured at pH 7.5 for 4 min with 5 μl of each suspension and 100 μl of each supernatant. (a) ATPase activity in the pellet plotted *versus* ATPase activity in the corresponding supernatant. 0, initial membranes; 1, 1 mM Tris; 2, 1 mM Tris, 0.1 mM Mg^{2+}; 3, 1 mM Tris, 1 mM Mg^{2+}; 4, 1 mM Tris, 5 mM Mg^{2+}; 5, 1 mM Tris, 10 mM Mg^{2+}; 6, 5 mM Tris; 7, 10 mM Tris; 8, 1 mM Tris, 10 mM KCl. (b) Results of depletion procedures 1 to 5 plotted *versus* Mg^{2+} concentration. △—△, activity in the supernatant; □—□, activity in the pellet.

TABLE I

RELEASE OF ATPase FROM MEMBRANE PARTICLES

The experimental procedure is described in Material and Methods. ATPase activity was measured at pH 7.5 (triethanolamine buffer) with 3.3 mM ATP and 1.66 mM Mg^{2+}.

Fraction	*Total protein (mg)*	*Total ATPase activity (μmoles P_i/min)*	*Specific activity (μmoles P_i/min per mg protein)*
Initial membrane suspension in 1 mM Tris–HCl buffer (pH 7.6) containing 1 mM $MgCl_2$	600	258	0.43
Depleted membrane	500	25	0.05
Solubilized fraction	100	140	1.40
$(NH_4)_2SO_4$ precipitate	78	134	1.70

pension, together with 1 mM Tris buffer. Release of 50% ATPase occurs when Mg^{2+} drops to approximately 0.5 mM. Fig. 1a also shows that increasing the ionic strength with either Tris–HCl or with KCl can partially prevent the release of ATPase but with less efficiency than Mg^{2+}.

Further experiments have shown that the release is substantially similar if the pH is varied from 7 to 9 or if 5–10% sucrose is present in the dilution buffer.

Kinetic and pharmacodynamic differences between membrane-bound and solubilized ATPase

The activity of solubilized ATPase was linear with protein concentration up to 25 μg/ml. The non-linearity above this protein concentration is not due to product inhibition and its reason is not understood, but might be attributed to interaction with other solubilized components. Since, in our preparation, ATPase is in a complexe mixture its description will be restricted to properties which can serve to differentiate soluble and membrane-bound ATPase

Fig. 2 shows the pH dependence of ATPase activities in native membranes, solubilized ATPase and a mixture of the latter with depleted membrane particles when the Mg/ATP ratio is 0.5 or 1. The pH optimum was shifted from approximately pH 8 to pH 9 by the solubilization, and the inhibition by excess Mg^{2+} was strongly enhanced.

Fig. 3 shows the ATP-concentration dependence and the inhibitory effect

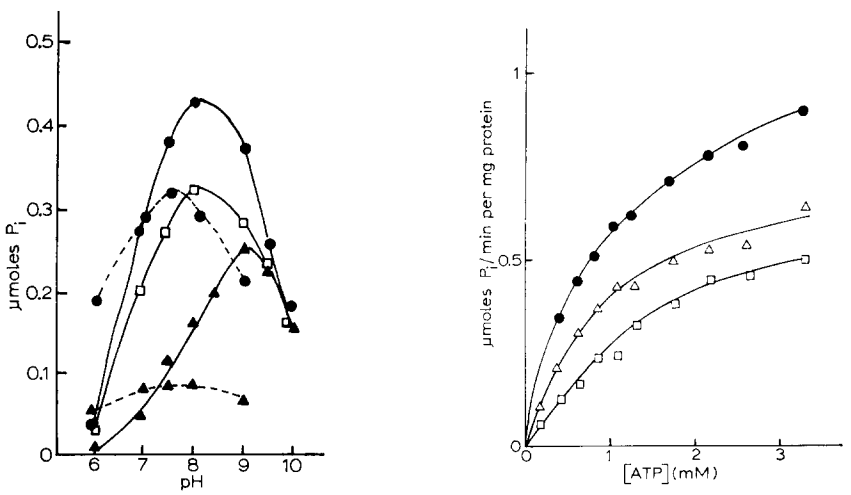

Fig. 2. pH dependence of ATPase activity in initial membranes, soluble ATPase, and a mixture of the latter with depleted membranes. The release of ATPase from membranes was described in the text. Experiments were carried out with 40 mM Tris–maleate buffer (pH 6–7.5), 40 mM triethanolamine buffer (pH 7.5–8.5), 40 mM Tris–HCl buffer (pH 8.5–9), 40 mM glycine–NaOH buffer (9.5–10), for 8 min with Mg/ATP=0.5 (smooth lines) and Mg/ATP=1 (dotted lines). ●—●, Initial membranes (112 μg protein); ▲—▲, soluble ATPase (18.5 μg protein); □—□, soluble ATPase (18.5 μg protein) mixed with depleted membranes (95 μg protein).

Fig. 3. Soluble ATPase activity *versus* ATP concentration in the presence of ADP. Experiments were carried out with 40 mM triethanolamine buffer (pH 7.5) and Mg/ATP=0.5. ●—●, ADP= zero; △—△, ADP=0.8 mM; □—□, ADP=1.9 mM.

of ADP in the soluble preparation. The K_m for ATP increased from 0.5 to 1 mM, and inhibition by ADP was roughly competitive with no obvious sigmoidicity as found in membrane-boundATPase[1].

Figs 4 and 5 show the action of DCCD and of Dio 9 on the membrane-bound and the soluble ATPase activities. DCCD hardly inhibited soluble ATPase in contrast to its dramatic action on membranes. The degree of inhibition of the soluble activity was somewhat variable from one preparation to the next. Dio 9 stimulated the soluble ATPase in contrast to its strong inhibition of membrane-bound ATPase. The stimulation by Dio 9 was also somewhat variable and seemed more pronounced at higher protein concentrations.

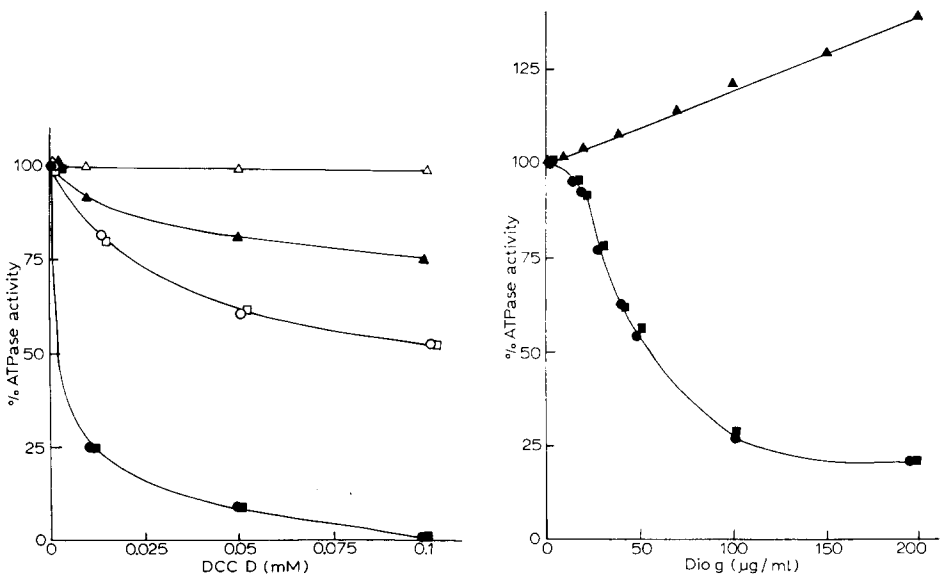

Fig. 4. Effect of DCCD on ATPase activity. Experiments were carried out with 40 mM triethanolamine buffer at pH 7.5. ●—●, membrane-bound ATPase; ▲—▲, soluble ATPase; ■—■, reconstituted ATPase. And with 40 M Tris–HCl buffer at pH 9. ○—○, membrane-bound ATPase; △—△, soluble ATPase; □—□, reconstituted ATPase.

Fig. 5. Effect of Dio 9 on ATPase activity. Experiments were carried out with 40 mM triethanolamine buffer at pH 7.5. Symbols as in Fig. 4.

As shown in Table II, some other inhibitors had similar effects on soluble and membrane-bound ATPase; inorganic orthophosphate and pyrophosphate and sodium azide inhibited both, and typical uncoupling agents were ineffective for both activities.

Reconstitution

As shown in Figs 2, 4 and 5, when soluble ATPase was mixed with depleted membrane particles, the properties of the mixture became similar to the original native membranes. Since Mg^{2+} was always present during ATPase assays, it is highly plausible that a membrane–ATPase complex was reformed. When reconstitution resulted in activation (neutral pH, high Mg^{2+}) and the Mg^{2+} – ATP solution

TABLE II

EFFECTS OF A VARIETY OF INHIBITORS

Experiments were carried out at pH 7.5 with 3.3 mM ATP and 1.66 mM Mg^{2+} with 20 μg of solubilized fraction or with 100 μg membrane.

Inhibitor	*Concn (mM)*	*Activity (% of control)*	
		Soluble	*Membrane bound*
Phosphate	1	75	76
	10	30	19
	20	0	8
Pyrophosphate	1	100	75
	5	75	41
	10	60	18
	20	10	0
2,4-Dinitrophenol	0.1	96	98
	0.5	98	90
Arsenate	0.5	105	100
	1	105	100
	10	100	100
Carbonyl cyanide *m*-chlorophenylhydrazone	0.01	100	100
	0.05	100	100
N-Ethylmaleimide	1	100	90
	2	100	80
Azide	1	8	30
	10	0	0

was added to start the reaction, it was rapid enough not to produce any detectable lag of ATP hydrolysis in spite of a greater dilution of the constituents as compared to the routine depletion prodecure. A lag of 30 s or more would have been detected.

Fig. 6 shows that the soluble ATPase activity became sedimentable after the addition of depleted membrane particles in the presence of Mg^{2+}, and again as in Fig. 1, the disappearance of non-sedimentable activity was linearly related to the appearance of sedimentable activity.

Similar experiments showed that the physical binding occurs at all pH values between 7 and 10.

It was important to assess qualitatively and quantitatively the contribution of the soluble and the particulate component to this reconstitution. An experiment of titration of one component with the other is presented in Fig. 7. When increasing amounts of soluble ATPase were added to different amounts of depleted membranes, the ATPase activity of the mixture (assayed at pH 7.5) increased by a factor of

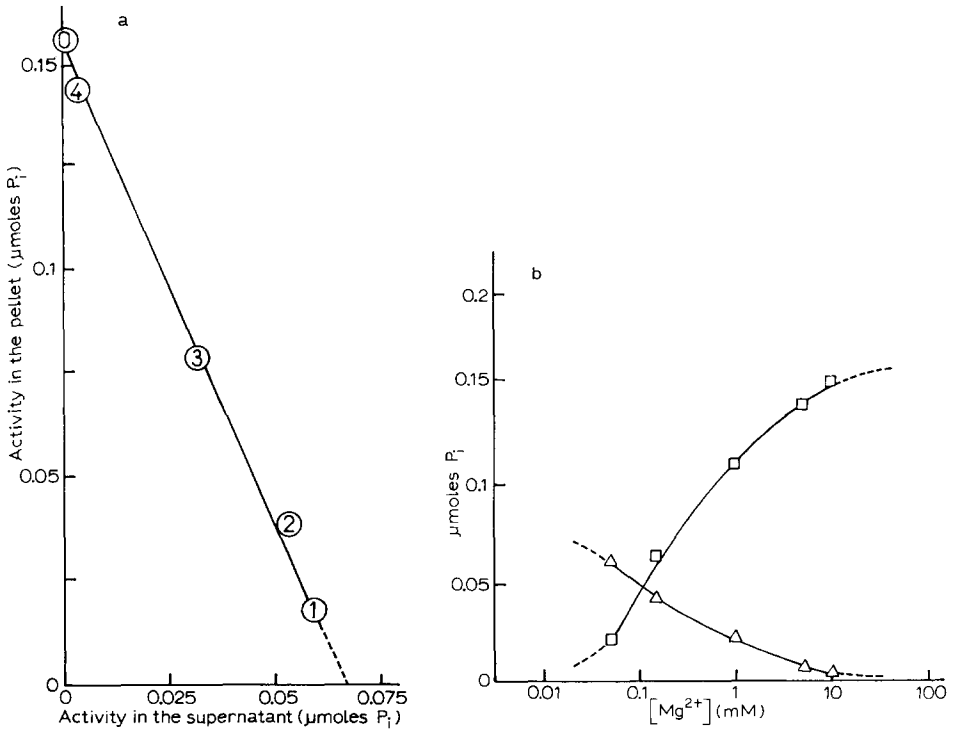

Fig. 6. Reconstitution of ATPase. Soluble ATPase and depleted membranes were prepared as described above. Soluble ATPase mixed with depleted membrane suspension in the proportion 100:5 (v/v) at variable final concentrations of Mg^{2+} at pH 7.5, was centrifuged 120 min at 165 000 × *g*. The pellet was resuspended in the original volume of depleted membranes with 1 mM Tris–HCl, pH 7.5. ATPase measurements were carried out at pH 7.5 with 100 μl of the supernatant and 5 μl of the pellet, respectively, for 4 min. (a) Activity in pellet plotted *versus* activity in the corresponding supernatant. 0, initial membranes; 1, 0 mM Mg^{2+}; 2, 0.1 mM Mg^{2+}; 3, 1 mM Mg^{2+}; 4, 10 mM Mg^{2+}. (b) Activity *versus* Mg^{2+} concentration. △—△, activity in the supernatant; □—□, activity in the pellet.

approximately 2.5 until an equivalence point was reached, above which addition of excess soluble ATPase resulted in a simple additivity of the activities, *i.e.* the residual slope of the curve of activity *versus* amount of soluble ATPase was identical to the slope observed in the absence of depleted membranes. At the equivalency point of several curves, the ratio of soluble ATPase to depleted membranes was constant. This also appears in Fig. 7b where the same data were plotted *versus* the amount of depleted membranes in the mixture.

When native membranes were mixed with soluble ATPase, the final activity was the algebraic sum of the activity of the two components as if such membranes had no available sites to fix the soluble enzyme. Therefore it seems that the depleted membrane particles provided more than a simple hydrophobic medium for the soluble ATPase to attain a different configuration with a different activity pattern. They seem to provide a limited number of specific sites for the ATPase molecules within which the appropriate interactions result in a complex change of pattern.

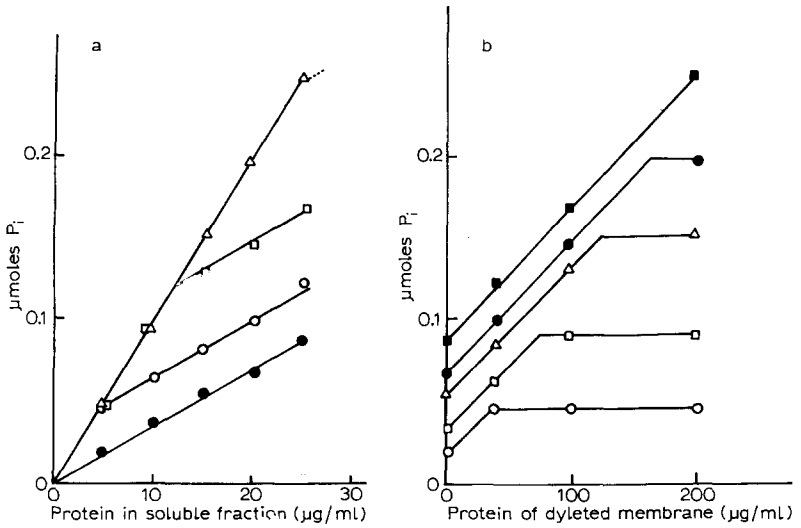

Fig. 7. Stoichiometry of reconstitution. The depleted membranes (Mbo) and soluble ATPase were obtained as described. The depleted membranes were resuspended in 1 mM Tris, pH 7.5, 5 mM $MgCl_2$. ATPase assays were carried out at pH 7.5. (a) Data plotted *versus* concentration of soluble fraction. ●—●, Mbo = 0; ○—○, Mbo = 40 µg; □—□, Mbo = 100 µg; △—△, Mbo = 200 µg. (b) The same data plotted *versus* concentration of depleted membranes. ○—○, solubilized ATPase = 5 µg; □—□, 10 µg; △—△, 15 µg; ●—●, 20 µg; ■—■, 25 µg.

The site of action of DCCD

Since DCCD is essentially inactive with respect to the soluble ATPase activity while it is strongly inhibitory towards the membrane-bound enzyme, the question could be raised of whether DCCD interacts primarily with the membrane sites and if so, whether this interaction abolishes the affinity of this site toward the soluble enzyme.

To approach this problem, the following experiment was performed. Native membranes were treated with DCCD to provide virtually complete inhibition, then the suspension was diluted in Mg^{2+}-free buffer and centrifuged. The activity in the pellet and in the supernatant was tested, and reconstitution was attempted between the two fractions and between each fraction and the relevant complementary fraction derived from a routine depletion procedure. The results are summarized in Table III. The DCCD-treated depleted membranes have lost the small residual activity found in ordinary depleted membranes, whereas the supernatant from the DCCD-treated membranes had an activity comparable to the control supernatant. This DCCD-treated soluble fraction gave a substantial reconstitution with control depleted membranes, whereas it lost all activity when mixed with the DCCD-treated depleted membranes. Similarly, these depleted membranes were able to inactivate the supernatant from the control. Therefore it was concluded that the DCCD used in the pretreatment remained associated with the particulate fraction, that this particulate fraction kept the capacity to bind soluble ATPase and that this association resulted in loss of activity.

Since inhibition by DCCD is virtually abolished at pH 9, (see Fig. 4), the

TABLE III

RECONSTITUTION OF SOLUBLE AND SEDIMENTABLE FRACTION OBTAINED FROM DCCD-TREATED MEMBRANES AND CONTROL MEMBRANES

Control depleted membranes and control soluble fractions were prepared as described in Material and Methods. DCCD-treated depleted membranes and DCCD-soluble fractions were described in the text. Activities were measured at pH 7.5 and at pH 9.

	Particulate fraction	*Soluble fraction*	*Activity at pH 7.5 (nmoles P_i/min)*	*Activity at pH 9 (nmoles P_i/min)*
1	Native (112 μg)		48	47
2	DCCD treated native (112 μg)		2.5	31.3
3	Control depleted (200 μg)		2	1.9
4	DCCD treated depleted (200 μg)		< 1	< 1
5		Control (18 μg)	12.5	31.3
6		DCCD treated (18 μg)	12	30
7	Control depleted (200 μg)	Control (18 μg)	44	43
8	Control depleted (200 μg)	DCCD treated (18 μg)	39	38
9	DCCD treated depleted (200 μg)	Control (18 μg)	2.5	31.3
10	DCCD treated depleted (200 μg)	DCCD treated (18 μg)	2	25
11		Supernatant from 9	< 1	< 1
12	Pellet from 9		1.25	25

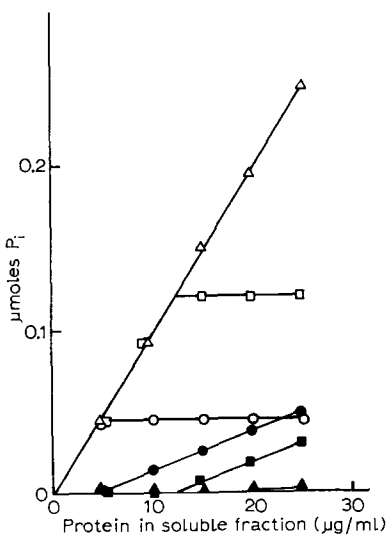

Fig. 8. ATPase activity *versus* soluble ATPase, in the presence of DCCD-treated membranes. The control depleted membranes (Mbo) were obtained as described in Material and Methods. The DCCD-treated depleted membranes (Mbo DCCD) were obtained as described in Table III. ATPase assays were carried out at pH 7.5. In mixtures containing both control and DCCD-treated depleted membranes, the latter was added last. ●—●, Mbo DCCD = 40 μg; ■—■, Mbo DCCD = 100 μg; ▲—▲, Mbo DCCD = 200 μg; ○—○, Mbo = 40 μg, Mbo DCCD = 200 μg; □—□, Mbo = 100 μg, Mbo DCCD = 200 μg ;△—△, Mbo = 200 μg, Mbo DCCD = 200 μg.

reconstitution of the membrane–enzyme complex at this pH was scrutinized by submitting the mixture to centrifugation.

The pellet of this second centrifugation exhibited activity at pH 9, and the supernatant was devoid of activity. Therefore, the binding of the soluble enzyme to membrane particles was obtained in spite of the presence of DCCD and in spite of the alkaline pH.

This provided the possibility of utilizing DCCD-treated depleted membranes as a specific inhibitor of soluble ATPase. It was checked that such particles had no inhibitory effect (in the short term) on the ATPase activity of native membranes. Once this point was established a reconstitution titration experiment, similar to that represented on Fig. 7, was performed, except that after mixing various amounts of depleted membranes and of soluble ATPase, Mg^{2+} was added, followed by an excess of DCCD-treated depleted membranes. The results of this experiment are represented in Fig. 8. In the lower part of Fig. 8 it can be seen that DCCD-treated depleted membranes have a definite equivalence with soluble ATPase similar in amounts to the equivalence point of ordinary depleted membranes, except that increasing the concentration of soluble ATPase to the equivalence point resulted in no activity, and excess soluble ATPase exhibited the usual activity. When DCCD-treated depleted membranes were added to a preformed mixture of reconstitution, they had no effect as long as depleted membranes were in excess of soluble ATP ase but inhibited the activity of soluble ATPase in excess above the equivalence point of the depleted membranes. This makes the determination of the equivalence point more accurate.

DISCUSSION

Membrane-bound ATPase of *E. coli* has been produced in soluble and purified form by previous authors. Evans[8] obtained solubilization by minute amounts of sodium dodecyl sulphate, Davies and Bragg[9] by an elaborate programme of washing with different solutions, and Kobayashi and Anraku[10] by a method closely related to the one described here.

Most descriptions are not detailed enough to pinpoint similarities or differences with our preparation. The essential new feature reported here is the production in a single step of membrane particles depleted of 90% or more of their initial ATPase activity and therefore suitable for reconstitution experiments. Soluble ATPase has many properties in common with membrane-bound ATPase: essential Mg^{2+} requirement at neutral pH, activation by Ca^{2+} and divalent ion-independent activity at higher pH. These results have not been reported in detail awaiting the purification of the enzyme. In contrast, a whole set of properties are different for the soluble enzyme: the pH-dependence curve shifted toward alkalinity by 1–1.5 pH units and the resistence was altered towards two of the most potent and most specific inhibitors of membrane ATPase, DCCD and Dio 9. This is strongly reminiscent of the loss of oligomycin sensitivity of mitochondrial ATPase[6] upon solubilization. In contrast, no clearcut cold sensitivity could be established. Similar solubilization–reconstitution was obtained with the membrane ATPase from other bacteria[11–14].

The reconstitution of membrane-bound ATPase reversed all explored allotopic

properties to the membrane bound pattern. No obvious intermediate states could be detected. The conditions of reconstitution seem to be very simple and trivial: a sufficient concentration of divalent cation. When this condition is fulfilled, the reconstitution proceeds instantaneously with high affinity and high specificity. No obvious non-specific binding was observed. The stoichiometry of this reaction permitted the titration of the ATPase-binding sites on the membrane. The number of sites found is slightly smaller than in the native membrane, while soluble ATPase is found in slight excess according to preliminary experiments, but these discrepancies are small enough not to seriously endanger the postulate that the binding sites are identical to the sites from which ATPase has been removed in the first place.

The ATPase-binding sites on the membrane can be titrated with soluble ATPase and behave therefore as a reactant in the reconstitution reaction, although they are doubtlessly a multicomponent part of the oxidative phosphorylation chain.

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