Effect of Salts on the Activity and Inhibition of *E. coli* Membrane ATPase by Ethacrynic Acid and Inhibitors

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Chaotropic Anions, *E. coli* ATPase, Ethacrynic Acid

The [Mg, Ca]-ATPase activity of *E. coli* depends on the anion present and follows the chaotropic sequence: Acetate$^-$ > HCO_3^- > Cl^- > I^- > NO_3^- > SCN^-. There are only small differences between the different alkali chlorides. The [Mg, Ca]-ATPase was inhibited by all these salts when the ratio of Mg or Ca to ATP was 1 : 5 at pH 9.1.

At pH 9.1 or 7.5 and a Mg to ATP ratio of 1 some salts activate and others inhibit the enzyme according to their position in the chaotropic sequence. With Ca the ATPase was activated under these conditions only by acetate. The contradictory activation or inhibition by NaCl or KCl reported by various authors are due to differences in the Mg : ATP ratios used. The inhibition of ATPase by ethacrynic acid, NEM, pCMB, DCCD and azide is much greater at pH 9.1 than at pH 7.5 and is drastically affected by NaCl or KCl.

In *E. coli* an ATPase which was slightly activated by Na^+ and K^+ and inhibited by strophanthin has been described [1, 2] but has not been confirmed by other workers [3]. This ATPase activity was so slight, however, that it could not be responsible for K^+ transport as is the case in animal cells [4]. We found a slight activation of the *E. coli* ATPase by NaCl at pH 7.5, but no inhibition by 10^{-3} M strophanthin [5]. Roisin and Kepes [6] reported slight inhibition of Na^+ and K^+ at pH 7.5. At pH 8.7 the activation of the ATPase produced by Na^+ or K^+ was greater, and the degree of activation depended on the anion present [2]. Evans [7], however, found an inhibition of the Mg-ATPase by Na^+ and K^+ in Tris HCl at pH 9.1 which was characterized as an allosteric inhibition [8]. The degree of inhibition was also dependent on the anion [9]. These differences were unsatisfactorily interpreted as being due to different strains of bacteria employed, growth conditions, or allotopy [9]. In the present series of experiments, however, we were able to show that these apparent discrepancies were produced by differences in the Mg : ATP ratio used by the various authors. The *E. coli* ATPase is involved in the anaerobic K^+ transport [10]. The K^+ transport in *E. coli* was inhibited by the diuretic ethacrynic acid [10] which inhibits animal ATPase [11]. The inhibition of K^+ transport was augmented by NaCl [10]. For comparison, we examined the effects of inhibitors and of salts on the ATPase of *E. coli*.

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Material and Methods

The culture of *E. coli* B 163 and its K^+-dependent mutant B 525 was described earlier [5]. Isolated cell membranes of *E. coli* B 163 and B 525 were prepared according to Evans [7].

ATPase determination

The ATPase was assayed at 37 °C in a shaking water bath in 1 ml samples with 5 mM Tris ATP [other conditions see legends]. The incubation time was 20 min. The protein content of the samples was held constant at 200 µg. Under these conditions the ATPase activity was linear with respect to time.

The reaction was stopped with 0.5 ml 15% cold TCA. After centrifugation, double determinations (0.5 ml each) of the inorganic phosphate in the supernatant were made according to Fiske and Subbarow [12]. The protein content was determined according to Lowry *et al.* [13].

Buffers

Tris-Cl buffers (with constant Tris concentration): Tris was titrated to the desired pH values and diluted to a Tris concentration of 0.2 M. Tris-Cl buf-

Abbreviations: NEM, *N*-ethylmaleimide; pCMB, *p*-chloromercuribenzoate; DCCD, *N,N'*-dicyclohexylcarbodiimide; ATPase, Mg, Ca dependent Adenosine Triphosphatase [E. C. 3.6.1.3]; Mg ATPase, Ca ATPase, [Mg, Ca] ATPase activity in presence of Mg or Ca respectively; Mg/ATP = 5/5, 5 mM Mg + 5 mM ATP.

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fers (with constant Cl^- concentration): HCl was titrated to the desired pH values with Tris, and diluted as indicated in Figs 1 and 3.

The chemicals were reagent grade. Tris ATP was obtained from Sigma, St. Louis, USA, pCMB from Serva, Heidelberg, DCCD and NaN_3 from E. Merck, Darmstadt, NEM from Fluka, Buchs, Switzerland. Ethacrynic acid was a gift from Dr. H. F. Hofmann, Sharp and Dohme, München.

Results

Dependence of ATPase activity on pH and ion concentration

Fig. 1 shows that the activity of the ATPase de-

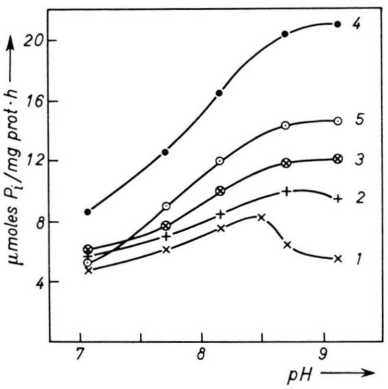

Fig. 1. pH-Dependence of Mg-ATPase. Preparation of *E. coli* B 163 in Tris buffers. Curve 1, Mg/ATP = 5/5, [Tris] = const.; 2, Mg/ATP = 5/5, [Cl'] = const.; 3, Mg/ATP = 5/5 = 0.3 M KCl, [Tris] = const.; 4, Mg/ATP = 1/5, [Tris] = const.; 5, Mg/ATP = 1/5 + 0.3 M KCl, [Tris] = const.

pends on the buffer used. In 0.1 M Tris-Cl buffers with constant Tris concentrations, a pH optimum was observed at pH 8.5 (Curve 1). In Tris-Cl buffers with constant (75 mM) Cl' concentrations, the activities are higher, especially in the alkaline buffers (Curve 2).

This suggests – as alkali cations are absent – that Cl^- activates the ATPase. The activity is increased by the addition of 0.3 M KCl (Curve 3). However, this activation only occurs when the concentrations of Mg and ATP are 5 mM. At a Mg:ATP ration of 1:5, the addition of KCl inhibits the ATPase (Curve 4 – 5) (compare also Figs 4, 6).

When Mg is replaced by Ca, the following phenomena are observed (Fig. 2): A pH optimum which is shifted toward higher pH (Curve 1), a sharp increase in activity which is displaced towards alkaline pH, when the ratio of Ca to ATP is 1:5 (Curve 4). KCl only inhibits the Ca-ATPase (Curve 2, 5). These results are identical to those of Evans [7, 9].

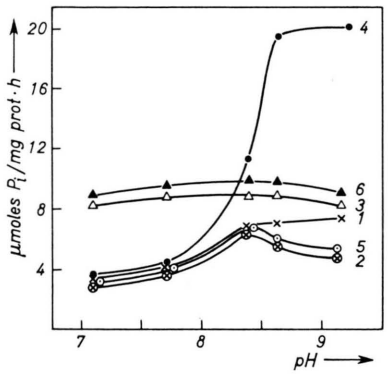

Fig. 2. pH-Dependence of Ca-ATPase of *E. coli* B 163 in Tris buffers with constant Tris-content. Curve 1, Ca/ATP = 5/5; 2, Ca/ATP = 5/5 + 0.3 M KCl; 3, Ca/ATP = 5/5 + 0.3 M Na acetate; 4, Ca/ATP = 1/5; 5, Ca/ATP = 1/5 + 0.3 M KCl; 6, Ca/ATP = 1/5 + 0.3 M Na acetate.

The addition of 0.3 M sodium acetate activates the Ca-ATPase at a Ca concentration of 5 mM (Curve 3) and activates or inhibits at 1 mM Ca dependent on pH (Curve 6). The Mg-ATPase is also activated by sodium acetate. The membrane preparations contain acetate kinase (E.C. 2.7.2.1), however, and the acetyl phosphate which is specifically synthesized from Mg, ATP, and acetate is largely hydrolysed under the experimental conditions (deproteinization with TCA and PO_4^{3-} determination). In control experiments, the activity of the acetate kinase was as high as 1/3 of the total Mg-ATPase activity in the presence of 0.3 M sodium actetate, so an activation of the Mg-ATPase by acetate was not demonstrated. The activities of the Mg-ATPase in the presence of sodium acetate reported by other investigators [1, 2, 9, 15, 16] using comparable ATPase preparations must be re-examined in light of this effect. Since the acetate kinase of *E. coli* is inactive with Ca (unpublished), the reported activation of the Ca-ATPase by sodium acetate is correct (Figs 2 and 5).

Effects of anions

In the preceeding section we suggested that the Mg-ATPase can be activated by chloride when Mg/ATP is 1. This is supported by the observation that the Mg-ATPase activity in Tris Cl-buffer increases with increasing buffer- and thus Cl^--concentration (Fig. 3, Curve 2). A similar effect was observed

with the mitochondrial ATPase [17, 18]. As shown in Fig. 3, this activation is only observed when the

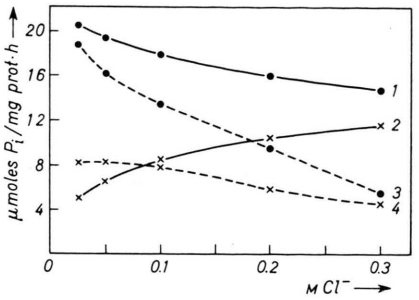

Fig. 3. Influence of Tris-Cl-concentration on Mg-ATPase (solid lines) and Ca-ATPase (broken lines) of the membrane preparation of *E. coli* B 163. Tris buffer pH 9.1 (constant Cl^--concentration with increasing concentration was used. Curve 1, Mg/ATP = 1/5; 2, Mg/ATP = 5,5; 3, Ca/ATP = 1/5; 4, Ca/ATP = 5/5.

ratio of Mg to ATP is 1. When the ratio of Mg or Ca to ATP is 1:5, increasing Cl^- concentration is

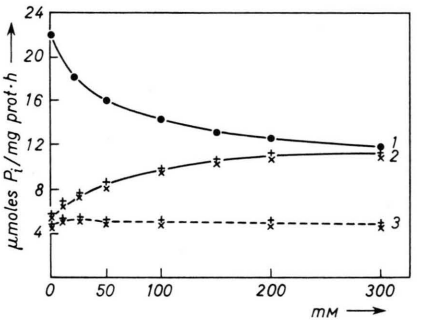

Fig. 4. Effect of salt concentration on Mg-ATPase of *E. coli* B 163 at pH 7.5 and 9.1 in Tris buffers with constant Tris-concentration. Curve 1, Mg/ATP = 1/5, pH 9.1, KCl; 2, Mg/ATP = 5/5, pH 9.1, KCl (×), NaCl (+); 3. Mg/ATP = 5/5, pH 7.5, KCl (×), NaCl (+).

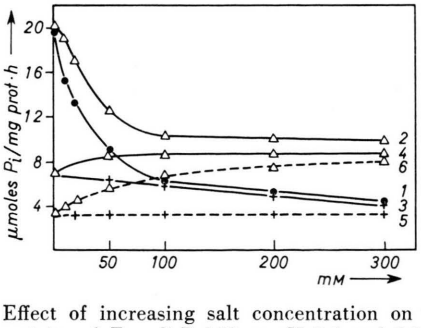

Fig. 5. Effect of increasing salt concentration on the Ca-ATPase activity of *E. coli* B 163 at pH 7.5 and 9.1 in Tris buffer with the same Tris-concentration. Curve 1, Ca/ATP = 1/5, pH 9.1, NaCl; 2, Ca/ATP = 1/5, pH 9.1, Na acetate; 3, Ca/ATP = 5/5, pH 9.1, NaCl; 4, Ca/ATP = 5/5, pH 9.1, Na acetate; 5, Ca/ATP = 5/5, pH 7.5, NaCl; 6, Ca/ATP = 5/5, pH 7.5, Na acetate.

accompanied by increasing inhibition which is stronger with Ca than with Mg.

The same result is observed with the addition of increasing concentrations of alkali chlorides (Figs 4 and 5), the alkali cations being slightly more effective than Tris Cl. So it is possible that the alkali cations are also somewhat active. The activation by alkali chloride is especially pronounced at pH 9.1. When a Hill plot of this activation at pH 9.1 is made (after subtracting the activity without salt), the value for n is 0.9.

To further characterize the effects of anions, we tested various salts at a concentration of 0.3 M. Table I confirms the results just described and also

Table I. Effect of various salts (0.3 M) on ATPase activity of *E. coli* B 163 with Mg ATP or Ca ATP in a concentration ratio of 5/5 or 1/5 respectively at pH 7.5 or 9.1 (µmoles P_i/mg prot·h).

	Mg			Ca		
pH	7.5	9.1		7.5	9.1	
Me/ATP (mM/mM)	5/5	1/5	5/5	5/5	1/5	5/5
ϕ	4.8	22.0	5.9	3.4	20.0	6.8
Na acetate	—	—	—	8.1	8.8	8.1
$NaHCO_3$	11.0	17.3	13.8	3.5	8.0	6.0
LiCl	4.8	13.8	10.5	3.4	5.1	4.0
NaCl	5.4	14.0	12.4	2.5	6.3	4.5
KCl	5.0	14.8	11.8	3.0	5.2	4.7
RbCl	5.2	14.3	11.9	3.4	5.3	4.6
CsCl	5.2	14.0	10.0	2.9	4.8	4.6
NaJ	4.2	7.4	9.5	2.9	3.1	2.5
$NaNO_3$	2.7	6.0	7.6	2.7	3.0	2.3
NaSCN	0.9	0.9	1.5	1.8	0.6	0.7

shows that the effects of the anions in the presence of Mg or Ca can be systematically arranged in a regular sequence. The values reflect activation and inhibition with respect to the control value (0.1 M Tris buffer, Tris concentration constant, no added salt). The ATPase activity in the presence of salts was found to decrease in the following order: $Acetate^-$, HCO_3^-, Cl^-, I^-,NO_3^-, SCN^-, which corresponds to a chaotropic sequence [19]. There are only slight differences between different alkali chlorides. SO_4^{2-} and F^- were not demonstrated due to their interference with Ca and Mg. Thus testing the ATPase in Tris SO_4 buffer with $MgSO_4$ [20] is not comparable with other conditions.

Effect of the Mg or Ca concentration on the ATPase

It was shown in the preceeding sections that the pH-dependence and the effects of salts on the activity depend on the ratio of Mg or Ca to ATP. Figs 6

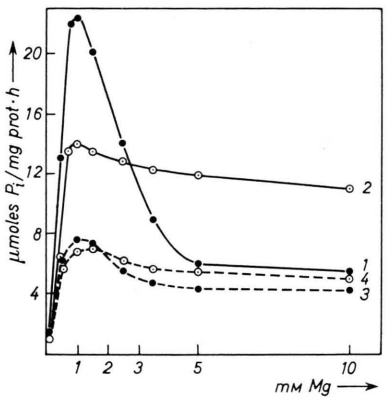

Fig. 6. Effect of Mg-concentration on ATPase activity of *E. coli* B 163 at pH 7.5 and 9.1 in Tris-Cl-buffer with the same Tris content. Curve 1, pH 9.1; 2, pH 9.1 + 0.3 M KCl; 3, pH 7.5; 4, pH 7.5 + 0.3 M KCl.

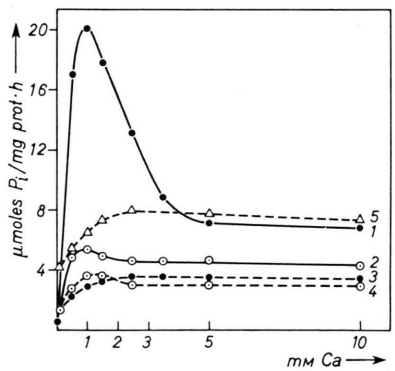

Fig. 7. Effect of Ca-concentration on ATPase. Conditions as in Fig. 6. Curve 1, pH 9.1; 2, pH 9.1 + 0.3 M KCl; 3, pH 7.5; 4, pH 7.5 + 0.3 M KCl; 5, pH 7.5 + 0.3 M Na acetate.

and 7 show the dependence of the (Mg, Ca)-ATPase activity on the concentration of Mg or Ca. At pH 9.1, and with an ATP concentration of 5 mM, a sharp peak in the activity is observed with 1 mM Mg or Ca (Curve 1). Above 5 mM Mg or Ca the (Mg, Ca)-ATPase activity decreases only a little further. In the presence of 0.3 M KCl or NaCl the peak at 1 mM Mg or Ca is suppressed (Curve 2). The level of the curve with 0.3 M KCl lies above the control without salt for Mg-ATPase (activation) and below it (inhibition) for Ca-ATPase. At pH 7.5 this relationship is small. The contradictory activation or inhibition by NaCl or KCl of *E. coli* ATPase [1, 2, 5, 6, 7, 9, 14] are thus due to differences in the Mg : ATP ratio (see Table II). This rule is also applicable to the ATPase of *S. aureus* [16], *S. faecalis* [21], *Micrococcus lysodeikticus* [22], *Bac. megaterium* [23] (Table II).

Table II. Activation and inhibition of various bacterial ATPases with respect to pH and Mg/ATP ratio.

pH	Mg/ATP	Effect of NaCl or KCl	Ref.
8,7	1	activation	1, 2
9.1	1 : 5	inhibition	7, 9
9.0	1 : 2.5	inhibition	14
7.5	1	slight activ.	5
7.5	1 : 2	slight inhib.	6
6.0 (Tris acet.)	1	slight activ.	16
7.5	1	slight activ.	21
7.5	1 : 2	slight inhib.	22
7.2	1 : 2	slight inhib.	23

Effects of ethacrynic acid and inhibitors

The effects of ethacrynic acid, azide and DCCD on the Mg-ATPase are shown in Figs 8 – 10. The

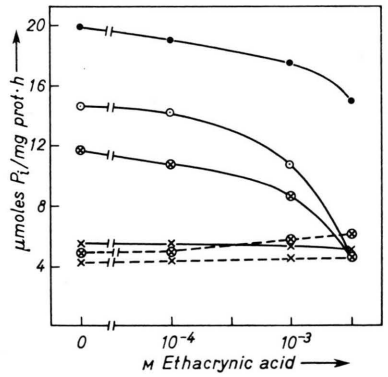

Fig. 8. Effect of ethacrynic acid on Mg-ATPase activity of *E. coli* B 163. Solid lines: pH 9.1, broken lines: pH 7.5. ●, Mg/ATP = 1/5; ⊙, Mg/ATP = 1/5 + 0.3 M KCl; ×, Mg/ATP = 5/5; ⊗, Mg/ATP = 5/5 + 0.3 M KCl.

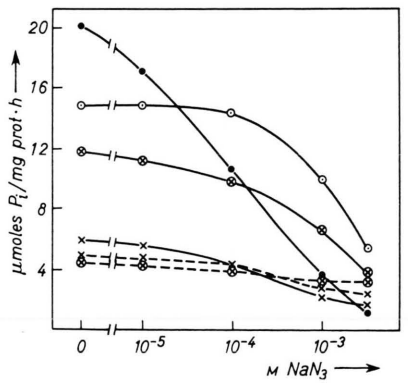

Fig. 9. Effect of azide on Mg-ATPase of *E. coli* B 163. Symbols as Fig. 8.

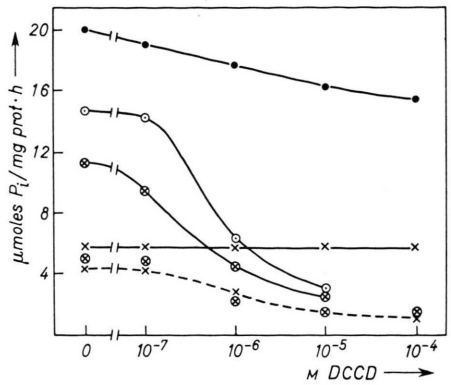

Fig. 10. Effect of DCCD on Mg-ATPase of *E. coli* B 163. Symbols as Fig. 8.

Ca-ATPase is affected in the same manner, although the control values are different (see Table I). The results for the Ca-ATPase were therefore not presented. In general, inhibitors are either less effective at pH 7.5 than at pH 9.1 (pCMB [not shown], azide and DCCD) or they do not inhibit at all at pH 7.5 (ethacrynic acid and NEM in agreement with [20], not shown). DCCD is still the strongest inhibitor of the ATPase at pH 7.5. The effects of inhibitors at pH 7.5 are not significantly influenced by KCl, at pH 9.1, however, alkali chlorides are effective. Three different patterns at pH 9.1 were seeen:

1. Ethacrynic acid and DCCD did not inhibit in media with 5 mM Mg and without KCl. When the ratio of Mg: ATP was 1 : 5, there was only slight inhibition of the ATPase. In the presence of 0.3 M KCl there was strong inhibition at both Mg-concentrations. As DCCD also inhibitis aerobic K^+-transport in the presence of NaCl which is independent of ATPase [10], DCCD is no specific ATPase inhibitor.

2. The definite inhibition by pCMB was not appreciably changed by KCl (not shown).

3. The inhibition of the ATPase by azide (Fig. 9) was reduced by KCl. Thus a quantitative comparison of the inhibitor effect by various workers is only possible when pH, salt- and buffer-concentration are identical.

Substrate specificity

The membrane preparation only splits ADP at 20% of the rate it does ATP. The splitting of ADP has the same pH-dependence and the same sort of sensitivity to salts and inhibitors as ATP hydrolysis. Hydrolysis of ADP by purified ATPase was reported by Giordano *et al.* [24], but Kobayashi *et al.* [14] and Roisin and Kepes [6] did not observe it. It is probable that the splitting of ADP is due to the presence of adenylate kinase (E.C. 2.7.4.3) in the membrane preparation.

ATPase activity in B 163 and the K-deficient mutant B 525

In agreement with earlier results [5], we found under all conditions no differences between the ATPase from the two strains.

Discussion

The experiments were performed with isolated membrane vesicles for better comparison with studies on the *E. coli* membrane ATPase by various authors, to clarify the discrepancies in the effects of salts and for comparison with K^+ transport experiments. We also wished to prevent allotopic changes which may occur when the ATPase is released from the membrane and a possible loss of ATPase because Bragg *et al.* [15] have separated ATPase activities from *E. coli* which may belong to separate enzymes.

Evans [7, 9] and Davies *et al.* [3] showed that the enzyme, in spite of several differences in the effects of Mg and Ca (pH dependence, inhibition by alkali chloride), is a (Mg, Ca)-ATPase. The activity in the intact cell, however, depends only on Mg. *E. coli* requires Mg and no Ca for growth, and therefore the Ca content of *E. coli* cells grown without addition of Ca is very low (unpublished). Since the concentration dependence of the ATPase activity on Mg and Ca is about the same, Mg must be the physiological activator.

Mg and Ca have a biphasic effect at weakly alkaline pH. The activity peak at 1 mM Mg or Ca (with 5 mM ATP) could be due to an effector action by the Mg or Ca. (Mn and Sr did not have this effect.) If this is so, low concentrations of Mg and Ca must bind to a center on the surface of the enzyme and activate it, while higher concentrations (to 5 mM) bind to another site and inhibit the enzyme. This inhibitory site is probably at the enzyme protein, since purified *E. coli* ATPase also had an activity peak at a Mg : ATP ratio of 1 : 5 [14]. This activity peak can be suppressed by adding salts. The anion is probably responsible, since different salts such as alkali chlorides or Tris chloride inhibit.

The same chaotropic sequence of the anions tested is observed with the Mg- and Ca-ATPase activities.

Activation or inhibition is due to a change in V_{max} at a constant K_m value (not shown). From this it could be inferred that the number of substrate sites or the velocity of the reaction at the active center is changed by the salts. The activation by salt follows Michaelis-Menten kinetics or an adsorption isotherm. This could mean that few salt molecules (anions) react reversibly with the enzyme. The reaction could be with a site on the protein which is especially reactive at alkaline pH. It is also possible that the chaotropic anion effect is due to a change in the lipophilic character of the water [19] or a change in its surface tension, and thus to a change in the conformation of the enzyme protein. This mechanism could further explain the varying effects of salts on inhibitors, whose reaction sites would be varyingly exposed by conformational changes.

Since the mitochondrial ATPase is also activated by anions [17, 18] one may ask if salts play some role in the function of ATPase with respect to oxidative phosphorylation and transport. Moreover it is possible that salts can modify the action of some substances like diuretics and inhibitors on membrane bound proteins.

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