Adenosine Diphosphate Binding to Sodium-plus-Potassium Ion-Dependent Adenosine Triphosphatase

THE ROLE OF LIPID IN THE NUCLEOTIDE-POTASSIUM ION INTERPLAY

By JØRGEN JENSEN and PAUL OTTOLENGHI

Institute of Physiology, University of Aarhus, DK-8000 Aarhus C, Denmark

(Received 10 September 1976)

Delipidated dogfish rectal-gland $\text{Na}^+ + \text{K}^+$ -ATPase ($\text{Na}^+ + \text{K}^+$ -dependent adenosine triphosphatase), almost devoid of hydrolytic activity, is able to bind about 2 nmol of ADP/mg of protein. The 'affinity' of delipidated enzyme for ADP is not affected by K^+ in concentrations that greatly decrease the 'affinity' of native $\text{Na}^+ + \text{K}^+$ -ATPase. The K^+ -sensitivity of the ADP binding is in part restored by relipidation with dioleoyl phosphatidylcholine.

$\text{Na}^+ + \text{K}^+$ -ATPase* binds ATP and ADP to specific sites (Nørby & Jensen, 1971; Jensen & Nørby, 1971; Hegyvary & Post, 1971; Kaniike *et al.*, 1973). Partial inhibition of the enzyme with ouabain causes a decrease in nucleotide-binding capacity equal to the amount of ouabain bound (Hansen *et al.*, 1971). This binding of adenosine nucleotides is extremely sensitive to K^+ in that the affinity of the enzyme for the nucleotides decreases with increasing concentrations of K^+ (Nørby & Jensen, 1971; Hegyvary & Post, 1971).

If the $\text{Na}^+ + \text{K}^+$ -ATPase of the spiny dogfish is delipidated reasonably thoroughly, it loses practically all its capacity for hydrolysing ATP and *p*-nitrophenyl phosphate (Ottolenghi, 1975). This inactivation could be due to removal of lipids that function to give the correct configuration to the substrate-binding site, or it could be due to impairment of steps subsequent to binding of substrate.

In an attempt to elucidate the cause of this inactivation, we have studied the binding of nucleotide (ADP) to delipidated $\text{Na}^+ + \text{K}^+$ -ATPase and the effect of K^+ on this binding.

Methods

Enzyme preparations

The preparations of crude membranes and delipidated enzyme as well as the assay of their *p*-nitrophenyl phosphatase and ATPase activities have been described (Ottolenghi, 1975).

Delipidated enzyme (with ATPase and *p*-nitrophenyl phosphatase activities of about 0.6 and 2 μmol of substrate hydrolysed/h per mg of protein respectively) is obtained in a medium containing

* Abbreviation: $\text{Na}^+ + \text{K}^+$ -ATPase, $\text{Na}^+ + \text{K}^+$ -dependent adenosine triphosphatase (EC 3.6.1.3).

about 350 mM-KCl as well as bile salts. Since the influence of K^+ on the binding of ADP was to be investigated, it was essential to remove the K^+ from the preparation. The enzyme was therefore precipitated from solution by lowering the pH to 6.0 (at 0°C). The precipitate (which also contains the bile acids) was washed twice by suspension and centrifugation in a medium containing glycerol (25%, v/v), 12 mM-imidazole and 10 mM-EDTA (imidazole salt), pH 6.0 at 0°C. At this low ionic strength the enzyme is insoluble even in the presence of bile salts. The enzyme was washed three more times with the same medium but at pH 7.6–7.7 (at 0°C) to remove bile salts, which are soluble at this pH. The final concentration of K^+ in enzyme preparations washed by this procedure was 10 μM or less.

Relipidated enzyme was prepared as follows: $\text{L}-\alpha$ -dioleoyl phosphatidylcholine (Serdary Research Laboratories, London, Ont., Canada) was dissolved at a concentration of 2 mg/ml in a medium containing glycerol (25%, v/v), EDTA (sodium salt, 40 mM, pH 7.4), deoxycholate (0.2%) and cholate (0.1%). To 95 mg of the original unwashed delipidated enzyme protein was added 20 mg of $\text{L}-\alpha$ -dioleoyl phosphatidylcholine. Thus relipidated, the enzyme was precipitated and washed as described for the lipid-free preparation.

The delipidated enzyme can be reactivated with dioleoyl phosphatidylcholine to ATPase and *p*-nitrophenyl phosphatase activities of 600 and 250 μmol of substrate split/h per mg of protein respectively. As initially prepared, the delipidated enzyme contained 350 mM-KCl, which had to be removed before undertaking the binding experiments shown in Figs. 2 and 3. The washing procedure used made the delipidated enzyme lose much of its capacity for reactivation by lipid. It also made the relipidated enzyme lose much of its activity. We have not been able to prevent or reverse these changes.

ATP binding to the enzyme

This was measured by a rate dialysis technique (Colowick & Womack, 1969; Nørby & Jensen, 1971), by using conditions essentially as described by Nørby & Jensen (1971), but with 25% (v/v) glycerol in the solvent.

ADP (disodium salt) was obtained from Merck, Darmstadt, Germany, and $[U\text{-}^{14}\text{C}]\text{ADP}$ was from The Radiochemical Centre, Amersham, Bucks., U.K. Before use they were both purified and converted into their Tris salts by ion-exchange chromatography (Nørby & Jensen, 1971).

Results

Fig. 1 shows a Scatchard-type plot of the binding of ADP to a crude membrane preparation from shark salt gland, and the effect of K^+ on this binding.

The non-linearity of the binding curves indicates that the system is not a simple one. Nevertheless, it is permissible to interpret the shift of the curves towards the left, with increasing K^+ concentrations, as a K^+ -induced decrease in 'affinity' of the enzyme for nucleotide. K^+ is without obvious effect on the nucleotide-binding capacity (intercept on the ordinate).

Fig. 2 shows the corresponding results obtained with a delipidated enzyme preparation almost devoid

Fig. 1. Scatchard-type plot of ADP binding to the ATPase in crude microsomal membranes that have never been exposed to detergent

Binding of ADP was measured in the absence of K^+ (a) or in the presence of $183\text{ }\mu\text{M-K}^+$ (b), $457\text{ }\mu\text{M-K}^+$ (c) or 7.32 mM-K^+ (d). The protein concentration used was 2.0 mg/ml . A shift of the lines to the left in a Figure is related to a decrease in the 'affinity' of the enzyme for the ligand, ADP. The intercept on the ordinate is equivalent to the concentration (μM) of ADP-binding sites.

Fig. 2. Scatchard-type plot of ADP binding to a delipidated ATPase preparation in the absence of K^+ (○) or in the presence of 7.32 mM-K^+ (●)

For ease of comparison with the other Figures, the line has been recalculated for a protein concentration of 0.81 mg/ml . The actual concentration used was 0.88 mg/ml .

Fig. 3. Scatchard-type plot of ADP binding to a delipidated preparation of ATPase that had been relipidated with dioleoyl phosphatidylcholine in the absence of K^+ (○) or in the presence of 7.32 mM-K^+ (●)

The lines have been recalculated for a protein concentration of 0.62 mg/ml . The actual concentration used was 0.74 mg/ml .

Table 1. *Catalytic activities and ADP-binding characteristics of membrane-bound, delipidated and relipidated Na⁺+K⁺-ATPase*

The 'affinity' is expressed as $1/[ADP_{free}]$ at half-saturation of the enzyme with ADP. The values given are relative to that for the membrane-bound enzyme.

Type of enzyme	Activities (μmol of substrate split/h per mg of protein)		ADP binding		
	<i>p</i> -Nitrophenyl phosphatase	ATPase	Capacity (nmol/mg of protein)	Relative 'affinity'	K ⁺ -sensitivity
Membrane-bound*	78–109	376–423	0.68–0.98	1	Very sensitive
Delipidated	2	0.6	1.94	$\frac{1}{2}$	Insensitive
Relipidated	110	240	2.53	1	Sensitive

* Range for five values.

of hydrolytic activity. The binding capacity of the enzyme for ADP was 1.94 nmol/mg of protein, and the 'affinity' of the enzyme for ADP was about half that of the native enzyme. But now K⁺ at a concentration (7.32 mM) that had enormous effect on the 'affinity' of the native enzyme for ADP has become totally ineffective.

Lastly, Fig. 3 shows the results obtained with delipidated enzyme relipidated with dioleoyl phosphatidylcholine. The ADP-binding capacity (2.53 nmol/mg of protein) remains similar to that of delipidated enzyme, but the K⁺-sensitivity of the nucleotide binding has been partially restored by the lipid. The 'affinity' for ADP returns to that shown by the native enzyme.

The results obtained with the above three preparations are summarized in Table 1.

Discussion

The difference in ADP-binding capacity between the membrane-bound and the delipidated enzyme is most likely due to the removal of non-ATPase protein during the delipidation procedure.

Although the enzymic activity of delipidated and relipidated enzyme is affected adversely by washing, the 'affinity' with which ADP is bound is still of the same order of magnitude as with the native enzyme. Further, the ADP-binding capacity of the delipidated and relipidated enzymes is the same as that of the purest preparations obtained in this laboratory (J. C. Skou, personal communication).

Therefore we feel justified in concluding that removal of so much lipid from Na⁺+K⁺-ATPase that the enzyme's hydrolytic activity is almost completely lost does not change the structure of that part of the enzyme that contains the nucleotide-binding site. The function of the lipid must reside elsewhere in the sequence of steps leading to the hydrolysis of ATP.

We cannot tell whether the binding of K⁺ to specific sites on the enzyme requires phospholipid. But, if K⁺ is bound to lipid-free enzyme, then the interplay between nucleotide and cation binding (seen in the untreated enzyme and in the relipidated enzyme) certainly has a phospholipid requirement.

Since relipidation with dioleoyl phosphatidylcholine partially restores both K⁺-sensitivity and catalytic activity, it is likely that the two phenomena are related. The absence of catalytic activity from the lipid-free enzyme could also be an expression of a more general loss of function of K⁺ from the intact enzyme system.

The situation is quite different from that shown to exist with D- β -hydroxybutyrate dehydrogenase, where the existence of the nucleotide-binding sites is dependent on the formation of a protein-phospholipid complex (Gazzotti *et al.*, 1974).

We thank Ms. Edith Møller and Ms. Lis Hygom for their painstaking technical assistance.

References

- Colowick, S. P. & Womack, F. C. (1969) *J. Biol. Chem.* **244**, 774–777
- Gazzotti, P., Bock, H. G. & Fleischer, S. (1974) *Biochem. Biophys. Res. Commun.* **58**, 309–315
- Hansen, O., Jensen, J. & Nørby, J. G. (1971) *Nature (London) New Biol.* **234**, 122–124
- Hegyvary, C. & Post, R. L. (1971) *J. Biol. Chem.* **246**, 5234–5240
- Jensen, J. & Nørby, J. G. (1971) *Biochim. Biophys. Acta* **233**, 395–403
- Kaniike, K., Erdmann, E. & Schoner, W. (1973) *Biochim. Biophys. Acta* **298**, 901–905
- Nørby, J. G. & Jensen, J. (1971) *Biochim. Biophys. Acta* **233**, 104–116
- Ottolenghi, P. (1975) *Biochem. J.* **151**, 61–66