Phosphorylation states of the ($\text{Na}^+ + \text{K}^+$)-transporting ATPase in preparations from lamb kidney and electric-eel (*Electrophorus electricus*) electric organ

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Phosphorylation states of the ($\text{Na}^+ + \text{K}^+$)-transporting ATPase were studied in highly purified preparations isolated from electric-eel electric organ and from lamb kidney. The steady-state level of phosphorylated lamb kidney enzyme, obtained by reaction with [γ - ^{32}P]ATP, was not appreciably reduced in the presence of ADP unless oligomycin was present. The phosphorylated form of the electric-eel electric-organ enzyme was reduced by at least 95% under the same conditions, suggesting that the E_1P state in the kidney enzyme is more transitory than that in electric organ. The level of phosphorylation from [^{32}P]P_i was higher in the lamb kidney preparation than in the electric-organ preparation, and the difference in stimulation of phosphorylation by ouabain in the two preparations was striking. Ouabain increased the level of phosphorylation by 35% in the kidney preparation and 734% in the electric-organ preparation. The E_2P state seems to be stabilized by ouabain in the latter preparation. These findings, as well as the different reactivities of the thiol groups to blocking reagents in these preparations, suggest that the tertiary structure in the enzyme isolated from these two sources is different.

In the preceding paper (Harris & Stahl, 1984), as well as in previous studies (Harris & Stahl, 1976, 1977, 1980; Stahl & Harris, 1979), we have examined thiol groups of the Na^+, K^+ -ATPase in purified preparations. The electric-eel electric-organ preparation can be isolated in the absence of detergents (Cantley *et al.*, 1978) and is particularly useful for studies on the interaction of lipids with the enzyme. The lamb kidney preparation, isolated by using detergent solubilization (Lane *et al.*, 1979), is very stable and large amounts can be readily obtained.

We observed that the lamb kidney and electric-eel (*Electrophorus electricus*) electric-organ Na^+, K^+ -ATPase enzymes had similar specific activities and thiol contents; however, the accessibility of the thiol groups to blocking agents differed significantly between the two preparations. In the

lamb kidney enzyme, 54 ± 5 nmol of thiol groups/mg of protein† were available to Nbs₂ in Tris buffer, and an additional 97 ± 8 could be measured in the presence of 1% SDS. This differs from the electric-eel preparation, where 93 ± 9 nmol of thiol groups/mg of protein were available to Nbs₂ in buffer and an additional 65 ± 8 nmol were found in the presence of SDS. This suggests significant differences in tertiary structure between the enzyme isolated from lamb kidney and electric-eel electric organ.

We have used these enzymes in the presence of specific ligands which favour a particular conformational state of the enzyme [Stahl & Harris, 1979; the preceding paper (Harris & Stahl, 1984)]. The present study was undertaken to verify that the ligand conditions used did lead to the expected conformational state of the enzyme and to gain further insight into structural and enzymic differences between the lamb kidney and electric-eel electric-organ enzyme preparations.

† The thiol-group values obtained for the preparations used for the present study were somewhat higher than those used in the preceding paper (Harris & Stahl, 1984).

Abbreviations used: Na^+, K^+ -ATPase, ($\text{Na}^+ + \text{K}^+$)-transporting ATPase; SDS, sodium dodecyl sulphate; Nbs₂, 5,5'-dithiobis(2-nitrobenzoic acid); CDTA, (1,2-cyclohexylenedinitrilo)tetra-acetate; Mops, 4-morpholinopropanesulphonic acid.

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Methods

Na^+, K^+ -ATPase was isolated from the electric organ of electric eel by the procedure of Cantley *et al.* (1978) and was stored in small portions in liquid N_2 . The enzyme was isolated from lamb kidney by the procedure of Lane *et al.* (1979) and could be stored at -2 – -4°C for several months without appreciable loss of enzyme activity.

Phosphorylation by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

This was carried out essentially by standard methods (Post *et al.*, 1969, 1975).

Enzyme (approx. 200 μg) was incubated for 10 s at 0°C in the presence of 100 mM-NaCl, 1 mM- MgCl_2 and 20 mM-imidazole, pH 7.4, in 600 μl . The phosphorylation was initiated at 0°C by the addition of 10–20 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (500–1000 c.p.m./pmol). The steady-state levels of phosphorylation (100% on ordinate; Fig. 1) were obtained by stopping the reaction after 5 s with 5 ml of ice-cold 5% (w/v) trichloroacetic acid containing 10 mM- H_3PO_4 . The mixture was immediately divided into three parts, and each was filtered on a 0.45 μm -pore-size filter (approx. 60 μg of protein/filter); the filter was washed three times with 2 ml of trichloroacetic acid/phosphate (see above), dried briefly and counted for radioactivity. This initial value was corrected for background by incubating one set in the presence of 100 mM-KCl instead of 100 mM-NaCl. To study dephosphorylation of the phosphoenzyme, 5 mM-CDTA or 5 mM-CDTA plus 2 mM-ADP or 5 mM-CDTA plus 50 μM -KCl were added 5 s after the addition of $[\text{P}^{32}]\text{ATP}$. Dephosphorylation was halted with trichloroacetic acid/phosphate after 5 s, and the phosphoprotein was collected, washed and counted for radioactivity as described above.

Phosphorylation by $[\text{P}^{32}]\text{P}_i$

Enzyme (approx. 750 μg) was preincubated for 10 min at 37°C in 50 mM-Mops/5 mM- MgCl_2 , pH 6.5, and with additions of 20 mM-KCl when indicated, in a final vol of 1 ml. The tubes were cooled to 20°C and phosphorylation was initiated by addition of 300 μl portions of the preincubated

filter. Filters were washed three times with 2 ml of ice-cold trichloroacetic acid/phosphate, and they were dried for 2 h at 100°C and counted for radioactivity.

$[\text{H}^3]\text{Ouabain}$ binding

This was performed by a modification of the method of Lindenmayer & Schwartz (1973). The purified enzyme was incubated for 20 min at 37°C in the presence of either 100 mM-NaCl/5 mM- MgCl_2 /2 mM-ATP/20 mM-Tris/HCl, pH 7.4, or 20 mM-Tris/HCl/2 mM-EDTA containing 0.1 mM- $[\text{H}^3]\text{ouabain}$. The binding was stopped by chilling the samples to 0°C and rapidly filtering on 0.45 μm -pore-size membrane filters, washed twice with the incubation media and once with 100 mM-Tris/HCl, pH 7.4. Each filter contained approx. 50 μg of protein. The filters were dried and counted for radioactivity in toluene-based scintillation cocktail {3 g of PPO (2,5-diphenyloxazole), 0.1 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] per litre of toluene}.

Results and discussion

The Na^+, K^+ -ATPase preparations isolated from kidney and electric organ had similar degrees of purity as judged by SDS/polyacrylamide-gel electrophoresis (results not shown). Both had Na^+, K^+ -ATPase activities of 1200–1700 μmol of P_i formed/h per mg of protein at 37°C and bound specifically 1500–2100 pmol of $[\text{H}^3]\text{ouabain}$ /mg of protein in the presence of Na^+ , Mg^{2+} and ATP (results not shown). In the lamb kidney enzyme the non-specific binding was 53% of the total ouabain binding, whereas, the non-specific component accounted for only 11% in the electric-organ preparation.

Dephosphorylation

A simplified sequential reaction scheme for the Na^+, K^+ -ATPase based on the work of Albers (1967) and Post *et al.* (1969) and others is as follows and probably involves several conformational changes:

enzyme to 200 μl of $[\text{P}^{32}]\text{P}_i$ (final concn. 0.1 mM; approx. 12×10^6 c.p.m./tube). After 20 min at 20°C the reaction was halted with 200 μl of ice-cold 25% trichloroacetic acid containing 90 mM- H_3PO_4 . This was followed by centrifugation at 5000 g for 15 min at 5°C . The supernatant was discarded and the pellet was resuspended in ice-cold 5% trichloroacetic acid containing 10 mM- H_3PO_4 and filtered on a 2.4 cm Whatman GF/A glass-microfibre

where the $\text{E}_1\text{-P}$ intermediate is considered a high-energy phosphate which in the absence of K^+ can react with ADP. The presence of the $\text{E}_1\text{-P}$ state can be operationally identified on the basis of this reactivity with ADP (Post *et al.*, 1969). The $\text{E}_2\text{-P}$ state does not react with ADP and decomposes in the presence of K^+ and is therefore distinct from $\text{E}_1\text{-P}$. Conversion of $\text{E}_1\text{-P}$ into $\text{E}_2\text{-P}$ can be blocked by oligomycin (Fahn *et al.*, 1966; Stahl,

1968). In the presence of P_i and Mg^{2+} the reaction to form $\text{E}_2\text{-P}$ is favoured (Post *et al.*, 1975) and the level of phosphorylation is raised in the presence of ouabain (Sen *et al.*, 1969).

The sensitivities of the phosphorylated intermediate(s) of the Na^+, K^+ -ATPase from lamb kidney and electric-eel electric organ to ADP and K^+ are shown in Fig. 1. The lamb kidney enzyme

Fig. 1. Dephosphorylation of purified Na^+, K^+ -ATPase from lamb kidney (a) and electric-eel electric organ (b). The phosphorylated enzyme was formed from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the Methods section in the absence (a, i and b, i) or presence (a, ii and b, ii) of $10\text{ }\mu\text{M}$ -oligomycin. At 5 s after addition of $[\text{}^{32}\text{P}]\text{ATP}$ the following were added: $\bullet$, 5 mM -CDTA; Δ , 5 mM -CDTA + 2 mM -ADP; $\square$, 5 mM -CDTA + 50 mM -KCl. Dephosphorylation was halted after 5 or 10 s. The results ($\pm$ s.d.) from single preparations of kidney and electric-organ enzyme assayed in triplicate in two to three separate experiments are shown. The mean steady-state levels of phosphorylation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of the lamb kidney and electric-eel electric-organ preparations were 2006 and $2425\text{ pmol of }[\text{}^{32}\text{P}]\text{phosphoprotein/mg of protein at }0^\circ\text{C}$ respectively.

phosphorylated intermediate was very sensitive to the presence of K^+ , but was only slightly decreased in the presence of ADP (Fig. 1a,i). This would indicate that the E_1 -P state is transitory and that the E_2 -P state predominates in the enzyme from this source under the conditions used. The opposite result was found for the Na^+, K^+ -ATPase prepared from electric eel (Fig. 1b). The phosphorylated intermediate in this case was rapidly dephosphorylated in the presence of ADP, presumably by reversal of the normal reaction scheme, with the phosphorylated enzyme donating the phosphate to ADP to form ATP. The presence of K^+ had only a slight effect on the spontaneous decay of the phosphorylated intermediate, indicating that the E_2 -P state was a minor constituent of the system compared with the E_1 -P state under these reaction conditions. When oligomycin was added to the lamb kidney Na^+, K^+ -ATPase, the phosphorylated intermediate was then sensitive to ADP and relatively insensitive to K^+ (Fig. 1a, ii). Thus oligomycin blocks the transformation from E_1 -P to E_2 -P and probably locks the enzyme from either source in this conformation.

In the presence of oligomycin (Fig. 1b, ii), the eel phosphorylated enzyme was more sensitive to ADP than to K^+ , with less than 2% of the phosphorylated species present after 5 s compared with approx. 5% without oligomycin.

Phosphorylation from P_i

The levels of phosphorylation of the Na^+, K^+ -ATPase preparations by the reverse route from P_i were different for the two preparations, as shown in Table 1. In the presence of Mg^{2+} only a very low level of incorporation of $[^{32}P]P_i$ into the electric-organ enzyme was achieved in comparison with the kidney enzyme. In accord with previous observations, incorporation of $[^{32}P]P_i$ was lower in the presence of K^+ (Post *et al.*, 1975). Addition of ouabain increased the level of phosphorylation by 35% in the kidney preparation and 734% in the

electric-organ preparation, and this phosphorylated form was not K^+ -sensitive. The stimulatory effect of ouabain is well established (Post *et al.*, 1975; Siegel *et al.*, 1969), but the quantitative difference between $[^{32}P]P_i$ incorporation into the two preparations in the absence of ouabain is striking. The levels of phosphorylation achieved by the reverse route are similar to the steady-state levels obtained by using $[\gamma\text{-}^{32}P]ATP$, especially for the lamb kidney enzyme. It seems likely that in the presence of ouabain a stable enzyme-phosphate-ouabain complex is formed in both preparations, so that a relatively similar level of phosphorylation is achieved. This complex is then stable to dephosphorylation even in the presence of K^+ (Table 1). However, the phosphoprotein level in the electric-organ preparation was always decreased by at least 25% by K^+ . This complex is probably important to understanding the mechanism of inhibition of the Na^+, K^+ -ATPase by cardiac glycosides, but specific details on the nature of this phosphorylated complex and other non-phosphorylated ouabain-enzyme complexes are lacking (Siegel *et al.*, 1969). In conclusion, the findings from these experiments allow us to predict that when using the lamb kidney Na^+, K^+ -ATPase, blockade with oligomycin is essential to assure that the E_1 -P state of the enzyme predominates in the presence of ATP, Mg^{2+} and Na^+ . In the presence of Mg^{2+} and P_i , a high steady-state level of phosphorylation and the E_2 -P is achieved, even in the absence of ouabain.

The enzymic characteristics and probable differences in tertiary structure between the Na^+, K^+ -ATPase purified from lamb kidney and electric organ likely involve developmental changes in portions of the enzyme not essential to the Na^+ - and K^+ -transporting function of the enzyme but necessitated by different intra- and inter-membrane associations within the cell membrane of individual cells. Interpolypeptide associations between the Na^+, K^+ -ATPase and other integral membrane proteins, for example with the insulin receptor (Resh, 1983), or with lysophosphatidylcholine acyltransferase (Szamel *et al.*, 1981), have been detected and such associations may be the basis for the tissue modifications in tertiary structure of the Na^+, K^+ -ATPase suggested in the present paper. A clearer understanding of these modifications will await further work with specific membrane probes and for a detailed structural analysis of the primary structure of the enzyme as isolated from different membranes.

References

- Albers, R. W. (1967) *Annu. Rev. Biochem.* **36**, 727-756
- Cantley, L. C., Jr., Gelles, J. & Josephson, L. (1978) *Biochemistry* **17**, 418-425

Table 1. Phosphorylation of Na^+, K^+ -ATPase from P_i . These results are from a single representative experiment carried out at 20°C for 20 min at pH 6.5 in quadruplicate as described in the Methods section.

Additions	$[^{32}P]P_i$ (pmol/mg of protein)	
	Lamb kidney	Electric-eel organ
$MgCl_2$ (5 mM)	1980	202
+ KCl (20 mM)	390	146
+ ouabain (1 mM)	2673	1684
+ ouabain (1 mM) + KCl (20 mM)	3061	1268

- Fahn, S., Koval, G. J. & Albers, R. W. (1966) *J. Biol. Chem.* **241**, 1882–1889
- Harris, W. E. & Stahl, W. L. (1976) *Biochim. Biophys. Acta* **426**, 325–334
- Harris, W. E. & Stahl, W. L. (1977) *Biochim. Biophys. Acta* **485**, 203–214
- Harris, W. E. & Stahl, W. L. (1980) *Biochem. J.* **185**, 787–790
- Harris, W. E. & Stahl, W. L. (1984) *Biochem. J.* **218**, 331–339
- Lane, L. K., Potter, J. D. & Collins, J. H. (1979) *Prep. Biochem.* **9**, 157–170
- Lindenmayer, G. E. & Schwartz, A. (1973) *J. Biol. Chem.* **248**, 1291–1300
- Post, R. L., Kume, S., Tobin, T., Orcutt, B. & Sen, A. K. (1969) *J. Gen. Physiol.* **54**, 3065–3265
- Post, R. L., Toda, G. & Rogers, F. N. (1975) *J. Biol. Chem.* **250**, 691–701
- Resh, M. D. (1983) *Biochemistry* **22**, 2781–2784
- Sen, A. K., Tobin, T. & Post, R. W. (1969) *J. Biol. Chem.* **244**, 6596–6604
- Siegel, G. J., Koval, G. J. & Albers, R. W. (1969) *J. Biol. Chem.* **244**, 3264–3269
- Stahl, W. L. (1968) *J. Neurochem.* **15**, 511–518
- Stahl, W. L. & Harris, W. E. (1979) in *Na^+, K^+ -ATPases: Structure and Kinetics* (Skou, J. C. & Nørby, J. G., eds.), pp. 157–167, Academic Press, London and New York
- Szamel, M., Schneider, S. & Resch, K. (1981) *J. Biol. Chem.* **256**, 9198–9204