

## Stabilizing Effects by $Mg^{2+}$ on Na,K-ATPase\*

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$Mg^{2+}$  is required for the activity of Na,K-ATPase.<sup>1,2</sup> It is required for the phosphorylation by ATP as well as by  $P_i$ .<sup>3,4</sup> When added before  $P_i$ ,  $Mg^{2+}$  was reported to inhibit phosphorylation by  $P_i$ .<sup>4</sup> In contrast, addition of  $Mg^{2+}$  early, or late, in the phosphorylation by ATP had no detectable effect on the initial rate of phosphorylation.<sup>3</sup> Addition of a high concentration of a chelator of divalent cations, 1,2-cyclohexylenedinitrilotetraacetic acid, to the phosphoenzyme decreased the rate of dephosphorylation.<sup>5</sup> This result may indicate that  $Mg^{2+}$  is bound to the phosphoenzyme and that it plays a role in the dephosphorylation step. Recently, evidence for an  $Mg^{2+}$ -induced conformational change at the ATP-binding site of the ATPase was demonstrated with a photoreactive ATP-analogue.<sup>6</sup> No direct studies on binding of  $Mg^{2+}$  to Na,K-ATPase have been reported.

The present paper describes two different conditions where  $Mg^{2+}$  is important in stabilizing the enzyme activity. The first condition is protection against inactivation of the dephosphoenzyme at acidic pH. The second condition is protection against inactivation by *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K) which is a reagent modifying carboxyl groups in proteins.<sup>7</sup>

**Experimental.** Na,K-ATPase was prepared from pig kidney as described by Jørgensen.<sup>8</sup> The sodium salt of ATP and Tris salt of *p*-nitrophenylphosphate (pNPP) were obtained from Sigma. The ATP was converted to its Tris salt as previously described.<sup>3</sup> *N*-Ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K) was purchased from Aldrich Chem. Comp., Inc. ATPase assay and the technique of testing the stability of the enzyme at various pH were previously described.<sup>9</sup> *p*-Nitrophenylphosphatase (pNPPase) was assayed in 3 mM  $MgCl_2$ , 10 mM KCl and 3 mM pNPP in 20 mM imidazole-HAc buffer, pH 7.5. Incubation volume was 0.5 ml. The reaction was stopped by the addition of 25  $\mu$ l of 50% trichloroacetic acid. One ml of 0.5 M Tris base was then added and the absorbance at 410 nm

was measured. Protein was measured according to the method of Lowry *et al.*<sup>10</sup> Chemical modification with Woodward's reagent K was carried out at 20 °C. About 10  $\mu$ g of enzyme protein in 175  $\mu$ l of 15 mM 2-morpholinoethanesulfonic acid (MES) buffer, pH 5.5, was incubated with 25  $\mu$ l of 5 mM Woodward's reagent K which was prepared immediately before use in 1 mM HCl. To the control 25  $\mu$ l of 1 mM HCl without the reagent was added. Exactly 2 min after the addition of Woodward's reagent K, 2.0 ml of cold 30 mM histidine buffer, pH 7.2, was added. Samples were then assayed for Na,K-ATPase and pNPPase.

**Results and discussion.** Na,K-ATPase was incubated at various pH at 30 °C for 60 min. The pH was then adjusted to 7.5 and the enzyme activity was assayed. At low pH the Na,K-ATPase activity and the pNPPase activity were inactivated (Fig. 1). At pH 4 only a few per cent of the original activities remained. At pH 5 the remaining activity was dependent on whether  $Mg^{2+}$  was present or not. Addition of 7.5 mM  $Mg^{2+}$  during the incubation at pH 5 protected the enzyme against inactivation.

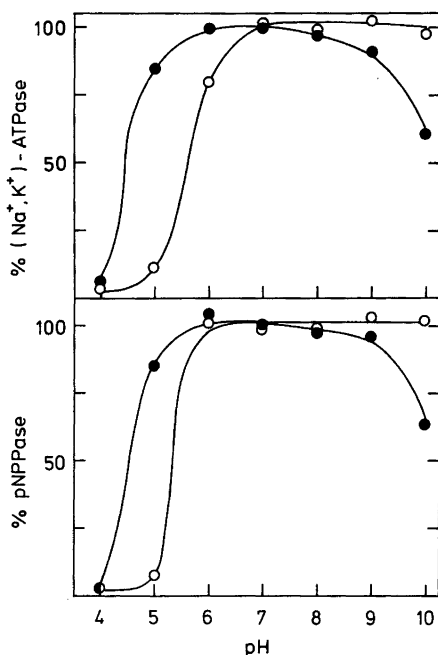


Fig. 1. Stability of Na,K-ATPase and pNPPase at various pH.  $MgCl_2$  was omitted (○), or 7.5 mM  $MgCl_2$  was included in the incubation mixture while the enzyme was treated at various pH (●). Final concentration of  $MgCl_2$  in both the Na,K-ATPase assay and in the pNPPase assay was 3 mM. Relative activities are presented.

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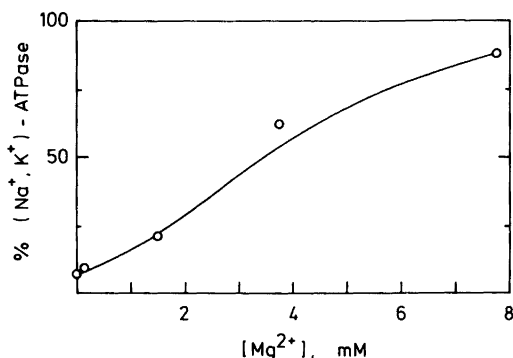


Fig. 2. Dependence of the stability of Na,K-ATPase at pH 5 on the concentration of Mg<sup>2+</sup> at 30° and pH 5 for 60 min. The pH was readjusted to 7.5 before assay of Na,K-ATPase.

About 85 % of the Na,K-ATPase and pNPPase was retained when Mg<sup>2+</sup> was present at pH 5 but only about 10 % in its absence. At alkaline pH the activities were stable in the absence of Mg<sup>2+</sup>. The presence of Mg<sup>2+</sup> made the activities more labile. In the presence of Mg<sup>2+</sup> about 60 % was retained after incubation at pH 10. The activities appeared to be stable between pH 6 and 8.

The ability of Mg<sup>2+</sup> to protect Na,K-ATPase and pNPPase was similar. Therefore the concentration dependence of Mg<sup>2+</sup> as a protective agent was tested on the ATPase activity only (Fig. 2). Relatively high concentrations of Mg<sup>2+</sup> were required. Half-maximal effect was observed at about 3.5 mM Mg<sup>2+</sup>.

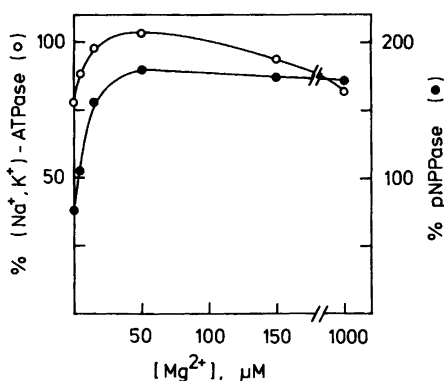


Fig. 3. Chemical modification of Na,K-ATPase by Woodward's reagent K. The enzyme was incubated with the reagent at various concentrations of Mg<sup>2+</sup>. After stopping the reaction with ice-cold 30 mM histidine buffer, pH 7.2, Na,K-ATPase and pNPPase were assayed.

This concentration is at least ten times higher than the  $K_{0.5}$  of Mg<sup>2+</sup> in the Na,K-ATPase reaction.<sup>11</sup>

After chemical modification of the enzyme with Woodward's reagent K under standard conditions in the absence of Mg<sup>2+</sup> about 75 % of the original Na,K-ATPase and pNPPase activities were retained (Fig. 3). In the presence of Mg<sup>2+</sup> protection against inactivation by the reagent was observed. At 50 μM Mg<sup>2+</sup> all activity assayed as Na,K-ATPase was retained. At a further increase of Mg<sup>2+</sup> the activity decreased again. At 1 mM Mg<sup>2+</sup> about 80 % of the original activity was retained. Similar to the effect on the Na,K-ATPase activity, Mg<sup>2+</sup> protected the pNPPase half-maximally at 10 μM concentration (Fig. 3). The activity of pNPPase, however, was not only protected against inactivation. At 50 μM Mg<sup>2+</sup> and higher concentrations, pNPPase reached activities which were about 180 % of the activity of enzyme not treated with the reagent. Previously, Woodward's reagent K has been used to modify carboxyl groups in proteins.<sup>7</sup> It is also known to act as a bifunctional cross-linking reagent.<sup>7</sup> A protection of Mg<sup>2+</sup> against inactivation by this reagent might be explained by protection of some essential carboxyl groups at the active center of the enzyme e.g. the carboxyl group of Asp which is phosphorylated by ATP.<sup>12</sup> The increase of activity as observed for pNPPase is, however, more difficult to explain. Maybe the combination of Mg<sup>2+</sup> and Woodward's reagent K resulted in the stabilization of a protein structure which has a high catalytic efficiency. Such a structure could be a functional dimer of α-subunits of the Na,K-ATPase with equally active (pNPPase) subunits in contrast to the native enzyme where these subunits might be out of phase.<sup>13</sup> Further experiments are necessary in order to find out the mechanism by which Mg<sup>2+</sup> and the reagent modify the enzyme. Since the effect of Mg<sup>2+</sup> was observed already at low concentrations, which were similar to the  $K_{0.5}$  for Mg<sup>2+</sup> in the Na,K-ATPase reaction, its observed effect probably involved binding to a site which normally binds the ion during hydrolysis of ATP. Contrarily, a high concentration of Mg<sup>2+</sup> was required in order to stabilize the enzyme at slightly acidic pH. This stabilization might be the result of a general stabilization of the protein structure in the membrane.

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1. Glynn, I. M. and Karlsh, S. J. D. *Annu. Rev. Physiol.* 37 (1975) 13.
2. Robinson, J. D. and Flashner, M. S. *Biochim. Biophys. Acta* 549 (1979) 145.

3. Mårdh, S. and Post, R. L. *J. Biol. Chem.* 252 (1977) 633.
4. Post, R. L., Toda, G. and Rogers, F. N. *J. Biol. Chem.* 250 (1975) 691.
5. Mårdh, S. *Biochim. Biophys. Acta* 391 (1975) 448.
6. Rempeters, G. and Schoner, W. *Eur. J. Biochem.* 121 (1981) 131.
7. Petra, P. H. and Neurath, H. *Biochemistry* 10 (1971) 3171.
8. Jørgensen, P. L. *Biochim. Biophys. Acta* 356 (1974) 36.
9. Mårdh, S. and Vega, F. *Biochim. Biophys. Acta* 601 (1980) 524.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* 193 (1951) 265.
11. Hexum, T., Samson, F., Jr. and Himes, R. *Biochim. Biophys. Acta* 212 (1970) 322.
12. Post, R. L. and Orcutt, B. In Naka, M. and Packer, L., Eds., *Organization of Energy Transducing Membranes*, Tokyo Univ. Press, Tokyo 1973, p. 35.
13. Repke, K. R. H. and Dittrich, F. In Skou, J. C. and Nørby, J. G., Eds., *Na,K-ATPase, Structure and Kinetics*, Academic, London and New York 1979, p. 487.

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