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Evidences for a Sulfhydryl Group in the ATP-Binding Site of ($Na^+ + K^+$)-Activated ATPase

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5,5′-Dithio-bis(2-nitrobenzoate) inhibited ($Na^+ + K^+$)-activated ATPase by affecting the Na^+-dependent phosphorylation reaction. ATP and ADP but not ITP protected the enzyme at low concentrations against the inactivation.

The ATP analogues 6-mercaptopurine riboside-5′-triphosphate and *S*-(2,4-dinitrophenyl)-6-mercaptopurine riboside-5′-triphosphate inactivated ($Na^+ + K^+$)-ATPase whereas the respective monophosphates left the enzyme unaffected.

S-(2,4-Dinitrophenyl)-6-mercaptopurine triphosphate inactivated the partial reactions of ($Na^+ + K^+$)-ATPase, *i.e.* the ADP-binding capacity and the Na^+-dependent phosphorylation reaction in parallel with the overall enzymatic activity. ATP protected against the inactivation of ($Na^+ + K^+$)-ATPase by *S*-(2,4-dinitrophenyl)-6-mercaptopurine triphosphate. Dithiothreitol hindered the inactivation of ($Na^+ + K^+$)-ATPase by *S*-(2,4-dinitrophenyl)-6-mercaptopurine riboside-5′-triphosphate and 6-mercaptopurine riboside-5′-triphosphate. Treatment with dithiothreitol restored the activity of ($Na^+ + K^+$)-ATPase which had been inactivated by 6-mercaptopurine riboside-5′-triphosphate but it did not restore the activity of that enzyme which was pretreated with *S*-(2,4-dinitrophenyl)-6-mercaptopurine riboside-5′-triphosphate.

S-(2,4-Dinitrophenyl)-6-mercaptopurine riboside-5′-triphosphate inactivated the associated enzymic activities of K^+-activated acetylphosphatase and K^+-dependent *p*-nitrophenylphosphatase but more slowly than ($Na^+ + K^+$)-activated ATPase.

It is concluded, that *S*-(2,4-dinitrophenyl)-6-mercaptopurine riboside-5′-triphosphate and 6-mercaptopurine riboside-5′-triphosphate exert their action on ($Na^+ + K^+$)-ATPase by reacting with a sulfhydryl group in the ATP-binding site of the enzyme.

($Na^+ + K^+$)-activated ATPase, which is involved in the active transport of Na^+ and K^+ through cell membranes [1,2], contains several sulfhydryl groups. Using radioactive *N*-ethylmaleimide, Hart and Titus [3] found 2 – 6 sulfhydryl groups alkylated per active center. The number of sulfhydryl groups alkylated varied with the nature of the ligands present during the alkylation procedure. The alkylation of –SH groups results in an inactivation of ($Na^+ + K^+$)-activated ATPase [4 – 9] [Eqns (1 – 4)] probably as a result of the inactivation of the partial reactions of the Na^+-dependent protein phosphokinase [8] [Eqn (1)], K^+-dependent phosphatase [8] [Eqn (3)] and of a blockage of conformational changes [5,7] [Eqn (2)]:

$$ATP + E_1 \overset{Mg^{2+},\,Na^+}{\rightleftharpoons} E_1 \sim P + ADP \qquad (1)$$

$$E_1 \sim P \overset{Mg^{2+}}{\rightleftharpoons} E_2 \sim P \qquad (2)$$

$$E_2 \sim P + H_2O \overset{K^+}{\rightleftharpoons} E_2 + P_i \qquad (3)$$

$$E_2 \rightleftharpoons E_1 \qquad (4)$$

The inactivation of ($Na^+ + K^+$)-activated ATPase by *N*-ethylmaleimide is partly prevented by the presence of ATP [4,9]. This protective effect of ATP

Abbreviations. clITP, 6-chloropurine riboside-5′-triphosphate; clIMP, 6-chloropurine riboside-5′-monophosphate; sIMP, 6-mercaptopurine riboside-5′-monophosphate, thioinosine monophosphate; sITP, 6-mercaptopurine riboside-5′-triphosphate, thioinosine triphosphate; Dnp-sIMP, *S*-[2,4-dinitrophenyl]-6-mercaptopurine riboside-5′-monophosphate; Dnp-sITP *S*-(2,4-dinitrophenyl)-6-mercaptopurine riboside-5′-triphosphate; Nbs_2, 5,5′-dithio-bis(2-nitrobenzoate), Ellman's reagent.

Enzyme. ($Na^+ + K^+$)-activated ATPase (EC 3.6.1.3).

could be caused by the formation of a distinct conformation of the enzyme (E_1) [3,4,8,10] resulting in a decrease of reactive −SH groups. An additional possibility would be an −SH group in the ATP-binding site which is protected against the alkylation by the ATP already bound.

ATP analogues reacting with sulfhydryl groups as affinity labels were helpful means to clarify this question. During the past few years, several protein-reactive ATP analogues have been synthesized showing these requirements [11−14]. We therefore studied the action of the ATP analogues clITP, sITP and Dnp-sITP on the activity of ($Na^+ + K^+$)-activated ATPase. The experiments reported here suggest that ATP interacts with an −SH group in the ATP binding site of ($Na^+ + K^+$)-activated ATPase.

MATERIALS AND METHODS

Chemicals

[^{14}C]ADP ammonium salt (spec. act. 487 Ci/mol) and [^{32}P]orthophosphate were from Amersham-Buchler (Braunschweig, Germany). [^{3}H]Ouabain (spec. act. 13 Ci/mmol) was from New England Nuclear (Dreieichenhain, Germany). Biochemicals and Nbs_2, *i.e.* 5,5′-dithio-bis-(2-nitrobenzoic acid), were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). 6-Chloropurine riboside and 6-Mercaptopurine riboside were from Pharma-Waldhof GmbH (Düsseldorf, Germany). DEAE-Sephadex A25 was from Pharmacia (Frankfurt). Dowex W50 was obtained through Serva (Heidelberg). All organic solvents were dried and stored over a molecular sieve (0.4 nm). All other chemicals were of analytical grade and obtained from E. Merck AG (Darmstadt, Germany) and Merck-Schuchardt (München, Germany).

Synthesis of 6-Chloropurine Riboside 5′-Monophosphate and 5′-Triphosphate. 6-Chloropurine riboside-5′-monophosphate was prepared according to Yoshikawa *et al.* [15]. To 1 mmol 6-chloropurine riboside, which had been dissolved in 3 ml triethyl phosphate, 2 mmol $POCl_3$ and 0.5 mmol H_2O were added under stirring. The reaction was terminated after 6 h stirring at room temperature by the addition of 10 ml H_2O. After adjusting the pH to 7.5 with triethylamine, the nucleotide monophosphate was precipitated as the barium salt. To the reaction medium, which had been filled up with water to a total volume of 100 ml, 4 ml of 1 M barium acetate were added followed by 400 ml ethanol. The barium salt was precipitated after 2 h standing at 0 °C by centrifugation and was washed twice with ethanol. The sediment was taken up in 300 ml H_2O and the solution was filtered. The filtrate containing the 6-chloropurine riboside-5′-monophosphate was poured on to a column (1.5 × 10 cm) of DEAE-Sephadex A25 (bicarbonate form). The column was washed with a linear gradient (0−0.3 M) of triethylammonium carbonate pH 7.5. The 6-chloropurine riboside-5′-monophosphate was eluted at about 0.1 M buffer concentration. Yield: 60%.

For the synthesis of the triphosphate according to Michelson [16], 1 mmol of the dry 6-chloropurine riboside-5′-monophosphate (free acid) was dissolved with about 10 ml dry dimethylformamide containing 1 mmol tributylamine and the solution was brought to dryness under vacuum in a rotatory evaporator. The residue was solubilized in 10 ml dry dimethylformamide and again evaporated. This process was repeated. The oily residue was taken up in 6 ml dimethylformamide, 12 ml dioxane, 1.2 ml tributylamine and 0.7 ml diphenylchlorophosphate. The mixture was allowed to stand at room temperature for 2 h. After evaporation under vacuum, the oily residue was treated with dry diethylether (40 ml) at 0 °C for 20 min. The ether was decanted and the oily residue was dried in the vacuum for a few minutes. The residue was taken up in a mixture containing 7.8 ml dimethylformamide, 0.8 ml tributylamine and 2.1 ml pyridine and added to 4 mmol of the tributylammonium salt of pyrophosphoric acid, prepared in the usual manner [17]. The reaction was allowed to proceed for 20 h in the dark with moderate stirring. The reaction was terminated by the addition of 40 ml H_2O. The mixture was extracted 4 times with 25 ml diethylether. The combined organic phases were washed 4 times with 5 ml H_2O. The combined water extracts were evaporated to dryness under vacuum and the residue taken up in a few ml of water. This procedure was repeated three times. Finally the residue was taken up in 50 ml of water and applied to a column (1.5 × 10 cm) of DEAE-Sephadex A25 in the bicarbonate form. The column was developed with a linear gradient of 0−0.6 M triethylammonium carbonate pH 7.5 (2000 ml). The 6-chloropurine riboside-5′-triphosphate was eluted at a buffer concentration of about 0.35 M. The compound having the same absorption spectrum as the 6-chloropurine riboside was synthesized with a yield of 30%. It gave a single spot in poly-(ethyleneimine)-cellulose thin-layer chromatography in 0.75 M KH_2PO_4 adjusted to pH 3.5 with HCl and it had the ratio of the amount of purine moiety to labile phosphate and total phosphate, respectively, to be expected for 6-chloropurine riboside-5′-triphosphate.

Synthesis of 6-Mercaptopurine Riboside 5′-Monophosphate and 5′-Triphosphate. 2′:3′-*O*-Isopropylidene-6-mercaptopurine ribonucleoside, which had been obtained from 6-mercaptopurine ribonucleoside in 65% yield according to Hampton and Maguire [18],

was converted to 6-mercaptopurine riboside-5'-phosphoric acid with 2-cyanoethyl phosphate according to Tener [19]. Yield 70%.

6-Mercaptopurine riboside-5'-triphosphate was synthesized in 60% yield from dry 6-mercaptopurine riboside-5'-phosphoric acid with method 2 of Murphy *et al.* [20]. Tributylammoniumpyrophosphate was prepared according to Moffat [17]. Fractionation of the reaction products of the synthesis occurred on a column (1.5 × 10 cm) of DEAE-Sephadex A25 by elution with a linear gradient 0–0.8 M of triethylammonium carbonate pH 7.5 (3000 ml).

The Synthesis of S-(2,4-Dinitrophenyl)-6-mercaptopurine Riboside 5'-Monophosphate and 5'-Triphosphate

This was performed according to Faust *et al.*[1] [14] with the following modifications.

S-(2,4-Dinitrophenyl)-6-mercaptopurine Riboside 5'-Monophosphate. 1 mmol of freshly prepared 6-mercaptopurine riboside-5'-phosphoric acid was solubilized in a mixture of 14 ml ethanol and 6 ml water at room temperature and adjusted to pH 7 by the addition of 2 N NaOH. To this mixture, which was refluxed at 60–70 °C, 0.62 ml of a 2 M ethanolic solution of 2,4-dinitro-1-fluorobenzene was added and the pH was maintained at pH 7–7.5 by the dropwise addition of 0.5 ml 2 N NaOH. The formation of the dinitrophenyl thioether of sIMP was terminated within some minutes. After cooling the reaction mixture, precipitating NaF was filtered off and the filtrate was extracted twice with diethylether. The water phase containing Dnp-sIMP was poured on to a column (2 × 12 cm) of Dowex W-50 (H^+-form). The Dnp-sIMP was eluted with water after, and well separated from, other remaining components. The yield of the lyophilized product was 60%. The spectrum and the R_F value on cellulose thin-layer chromatography were identical with the Dnp-sIMP of Faust *et al.* [14].

S-(2,4-Dinitrophenyl)-6-mercaptopurine Riboside 5'-Phosphomorpholidate. This was prepared from the freshly prepared dry *S*-(2,4-dinitrophenyl)-6-mercaptopurine riboside-5'-phosphate according to Moffat and Khorana [21] as reviewed in [22]. Yield 67%.

S-(2,4-Dinitrophenyl)-6-mercaptopurine Riboside 5'-Triphosphate. For the synthesis of this compound 0.4 mmol of the dry morpholidate were dissolved in 16 ml dimethylsulfoxide and added to 1.6 mmol tributylammonium salt of pyrophosphoric acid prepared as usual [17]. The solution was allowed to react in the dark for 2 days at room temperature. After addition of 120 ml water, the mixture was transferred to a column (1.5 × 10 cm) of DEAE-Sephadex A25 (bicarbonate form). The elution of the triphosphate was performed by a linear gradient 0–0.8 M triethylammonium carbonate pH 7.5 (3000 ml). Yield 60%.

Terminally-Labelled [^{32}P]ATP

[γ-^{32}P]ATP was prepared according to Glynn and Chappel [23].

Enzyme and Assays

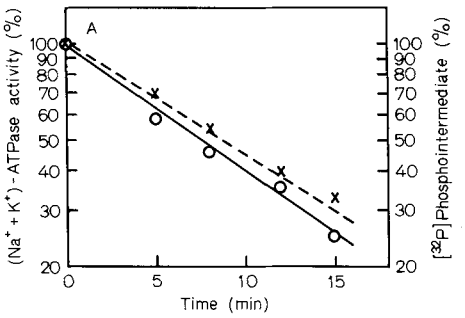
($Na^+ + K^+$)-activated ATPase from beef brain was prepared as described previously [24]. The enzymatic activity was measured with the coupled optical assay [24]. The reaction was continuously recorded and corrected for a Mg^{2+}-activated ATPase by inhibition of ($Na^+ + K^+$)-activated ATPase with 0.1 mM ouabain. One enzyme unit is defined as the amount of enzyme hydrolyzing 1 µmol ATP per min at 37 °C. Protein was estimated by the procedure of Lowry *et al.* [25]. The ADP-binding capacity of ($Na^+ + K^+$)-ATPase at 0 °C which corresponds to the ATP binding capacity was measured with EDTA-washed enzyme preparations as reported earlier [26,27]. Measurements of the ability of ($Na^+ + K^+$)-ATPase to form a [^{32}P]phosphointermediate were carried out according to the procedure of Post *et al.* [28] with minor modifications [29]. [^{3}H]Ouabain binding at 37 °C was measured in a modification [29] of the procedure of Matsui and Schwartz [30].

K^+-Activated Acetylphosphatase. 0.07–0.23 mg enzyme protein was incubated with 50 mM imidazole-HCl pH 7.6, 10 mM acetylphosphate (potassium, lithium salt), 5 mM $MgCl_2$ in a total volume of 1 ml at 37 °C for 10 min. Blanks contained 0.1 mM ouabain. The remaining acetylphosphate was determined as acetylhydroxamate by the addition of 1 ml hydroxylamine and 3 ml $FeCl_3$ solution [52].

K^+-Activated p-Nitrophenylphosphatase. 0.02–0.06 mg enzyme was incubated at 37 °C for 10 min in 1-ml volume with 50 mM imidazole-HCl pH 7.4, 4.5 mM *p*-nitrophenylphosphate (Tris salt), 5 mM $MgCl_2$ and 10 mM KCl. The reaction was terminated by the addition of 1 ml 1 M NaOH. The *p*-nitrophenol liberated was determined at 405 nm and corrected for a K^+-independent phosphatase by the omission of K^+ in the reference assay.

Inactivation of ($Na^+ + K^+$)-ATPase with ATP Analogues. 1–2 mg enzyme protein (spec. act. 1–4 U/mg) was incubated in a total volume of 0.5 ml at 30 °C or 37 °C with 100 mM buffer of varying pH

[1] We thank Dr U. Faust and Prof. H. Fasold (Frankfurt) for making available to us their preparation method for Dnp-sITP prior to its publication and also for their generous gift of Dnp-sITP for preliminary studies on ($Na^+ + K^+$)-activated ATPase. Our thanks are also due to Mr Rack (Frankfurt) who directed our attention to the preparation method for nucleotide triphosphates with diphenylchlorophosphate [16].



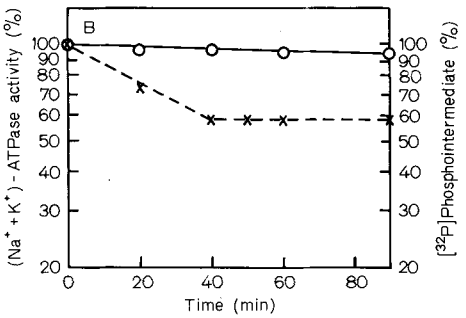


Fig. 1. *Comparison of the actions of Nbs_2 and* N-*ethylmaleimide on $(Na^+ + K^+)$-ATPase and the Na^+-dependent phosphoprotein formation.* (A) Effect of Nbs_2: 40 mg $(Na^+ + K^+)$-ATPase protein (spec. act. 3.0 U/mg) was incubated at 37 °C in 100 mM imidazole-HCl pH 7.25 and 133 μM Nbs_2 (total volume 15.0 ml). At the times indicated aliquots of 2.0 ml were withdrawn and centrifugated at 40000 × *g* for 60 min, after dilution with 5 ml cold 0.05 M cysteine + 20 ml H_2O. Na^+-dependent phosphorylation and determinations of $(Na^+ + K^+)$-ATPase activity and of protein were performed with the sediment which had been homogenized in 2 ml H_2O. (B) Effect of *N*-ethylmaleimide: 27 mg $(Na^+ + K^+)$-ATPase protein was incubated at 37 °C with 90 mM Tris-HCl pH 8.0 and 100 μM *N*-ethylmaleimide (total volume 10.0 ml). Aliquots were taken at the times indicated for determinations of Na^+-dependent phosphorylation (O——O) and $(Na^+ + K^+)$-ATPase activity (×----×)

and the ATP analogues indicated in the legends of the figures. Inactivation of $(Na^+ + K^+)$-ATPase was followed by transferring a 0.03-ml aliquot of the reaction medium to the coupled optical assay [24].

RESULTS

Studies with 5,5'Dithio-bis(2-nitrobenzoate)

In order to ascertain that sulfhydryl groups are involved in the first reaction of $(Na^+ + K^+)$-activated ATPase, we studied the effects of Nbs_2 and *N*-ethylmaleimide on the overall reaction [Eqns (1 − 4)] and the ability of the enzyme to form an Na^+-dependent phosphorylated intermediate [Eqn (1)]. In agreement with previous results [5 − 7], *N*-ethylmaleimide inhibited $(Na^+ + K^+)$-activated ATPase, whereas the ability of the enzyme to form a phosphorylated intermediate was unaltered (Fig. 1 B). Nbs_2, however, blocked both reactions (Fig. 1 A). This finding demonstrates, in agreement with previous studies [4 − 9], that −SH groups are involved in the Na^+-dependent protein phosphokinase reaction. Apparently the different sulfhydryl groups of $(Na^+ + K^+)$-activated ATPase have different sensitivities towards sulfhydryl reagents. ATP and ADP but not ITP protected the enzyme at low concentrations against the inactivation by Nbs_2 (Fig. 2). AMP, which binds with low affinity to the nucleotide binding site [31, 32] and *p*-nitrophenylphosphate could not protect against the inactivation by Nbs_2.

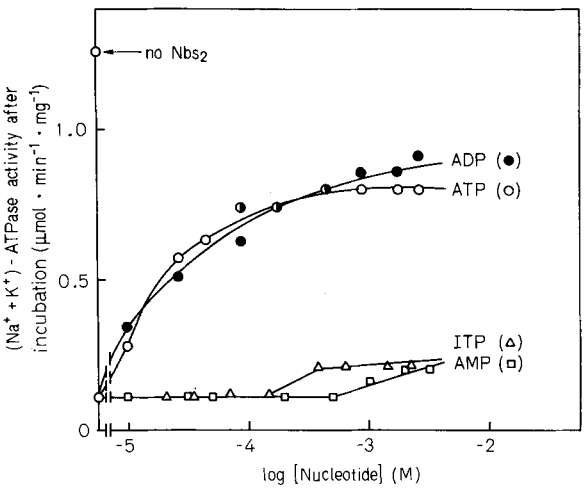


Fig. 2. *Effect of nucleotides against the inactivation of $(Na^+ + K^+)$-ATPase by Nbs_2.* 1.4 mg $(Na^+ + K^+)$-ATPase protein was incubated in a total volume of 1.0 ml at 37 °C for 35 min with 60 mM imidazole-HCl pH 7.25, 250 μM Nbs_2 and the nucleotides indicated

Determination of the Affinity of ATP Analogues for the ATP-Binding Site of $(Na^+ + K^+)$-Activated ATPase

The protective effect of ATP and ADP against the inactivation of the $(Na^+ + K^+)$-activated ATPase by Nbs_2 could be explained by an interaction of the 6-amino group of ATP with an −SH group of the enzyme as has been suggested to occur in myosin [13, 33]. This kind of interaction would hinder the access of Nbs_2 to an −SH group in the ATP-binding site. The protective effect of ATP, however, could also be explained by a conformational change of the enzyme concealing those −SH groups which are more superficial in the absence of ATP. In order to discriminate between the two possibilities, the protein-reactive ATP analogues clITP, sITP and Dnp-sITP were synthesized. Fig. 3 demonstrates that these ATP analogues compete with [^{14}C]ADP for the nucleotide-binding site. It has been shown previously, that the number of the ADP-binding sites of $(Na^+ + K^+)$-ATPase is

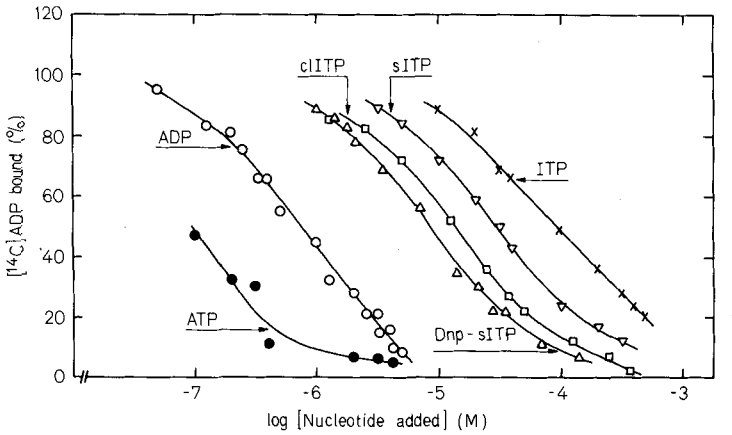


Fig. 3. *Competition between [^{14}C]ADP and other unlabelled nucleotides for binding to ($Na^+ + K^+$)-ATPase.* 103 pmol [^{14}C]ADP were mixed at 0 °C in polypropylene tubes with 1 mg EDTA-washed ($Na^+ + K^+$)-ATPase (spec. act. 2.5 U/mg), 50 mM imidazole-HCl pH 7.25 and the ATP analogues shown (total volume 2 ml). After sedimentation of the membranes at 70000 × *g* for 30 min at 4°C, the radioactivity was determined in the sediment. The control without additional nucleotides was taken as 100%. For details see Methods

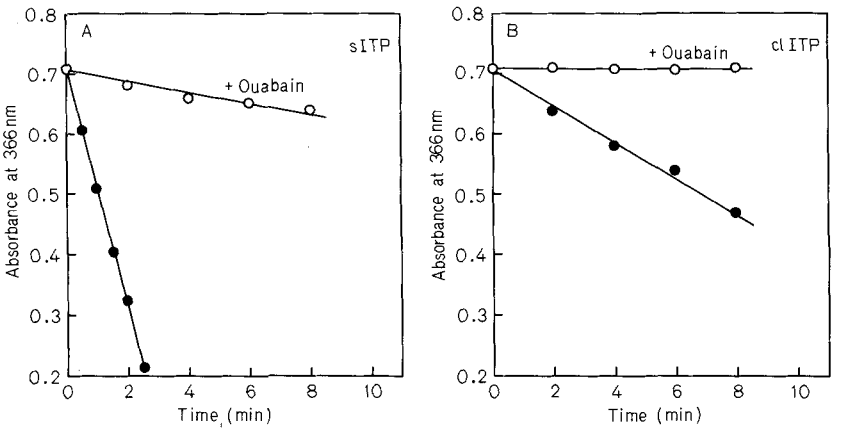


Fig. 4. *Hydrolysis of sITP and clITP by ($Na^+ + K^+$)-ATPase in the presence (○) or absence (●) of 0.1 mM ouabain.* The hydrolysis was followed with the coupled optical assay [24]. The assays contained instead of ATP in (A) 2 mM sITP; (B) 1 mM clITP

directly proportional to the specific enzymic activity of ($Na^+ + K^+$)-activated ATPase [26]. The affinity of the nucleotide triphosphates for the nucleotide-binding site, calculated according to Jensen and Nørby [32] from these data, are listed in the Table. It is evident that the ATP analogues have a lower affinity for the nucleotide binding site than ATP or ADP but have a higher affinity than ITP. ITP is a substrate of ($Na^+ + K^+$)-activated ATPase [34] and so are clITP and sITP substrates of the enzyme (Fig. 4). The hydrolysis of Dnp-sITP by ($Na^+ + K^+$)-activated ATPase was difficult to demonstrate. However, since [^{3}H]ouabain is bound *via* the Na^+-dependent pathway in the presence of Dnp-sITP (Fig. 5), it is highly probable that Dnp-sITP is a substrate of ($Na^+ + K^+$)-activated ATPase. Na^+ ions increase ouabain binding only under those conditions in which a phosphorylated intermediate is formed [35, 36]. The lower level of

Table 1. *Affinity of the ATP-binding site of ($Na^+ + K^+$)-ATPase for ATP analogues*
The dissociation constant K_d was calculated from the data in Fig. 3 by the equations given by Jensen and Nørby [32]

Nucleotide	K_d
	M
ATP	1×10^{-7}
ADP	5×10^{-7}
Dnp-sITP	5×10^{-6}
clITP	9×10^{-6}
sITP	2×10^{-5}
ITP	6×10^{-5}

[^{3}H]ouabain bound is the result of a change of affinity of the ouabain receptor during the inactivation of the enzyme (H. Pauls, R. Patzelt-Wenczler and W. Schoner, unpublished experiments).

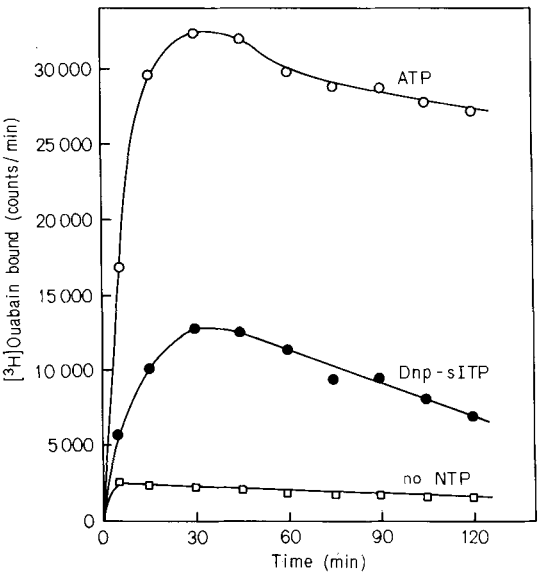


Fig. 5. *ATP and Dnp-sITP-supported ouabain binding to $(Na^+ + K^+)$-ATPase via the Na^+-dependent pathway [51].* 0.37 mg $(Na^+ + K^+)$-ATPase protein was incubated at 37 °C for the times indicated with 50 mM imidazole-HCl pH 7.25, 3 mM $MgCl_2$, 100 mM NaCl, 15 pmol [^{3}H]ouabain and 0.1 mM nucleotide triphosphate in a total volume of 2 ml. For details see Methods

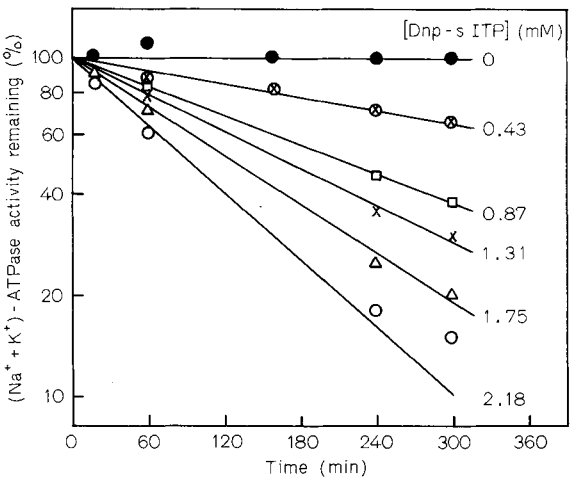


Fig. 6. *Inactivation of $(Na^+ + K^+)$-ATPase from beef brain by increasing concentrations of Dnp-sITP.* 0.45 mg protein of $(Na^+ + K^+)$-ATPase (spec. act. 1.8 U/mg) were incubated with 200 mM Tris-HCl pH 8.2 and the indicated concentrations of Dnp-sITP at 30 °C (total volume 0.5 ml)

Inactivation of $(Na^+ + K^+)$-Activated ATPase by Dnp-sITP

Incubation of $(Na^+ + K^+)$-ATPase with Dnp-sITP resulted in a time-dependent and concentration-dependent inactivation of $(Na^+ + K^+)$-activated ATPase (Fig. 6). The inactivation half-life calculated at infinitely high concentrations of Dnp-sITP corresponds to a value of 30 min (Fig. 7). The intercept on the horizontal axis is $-0.15 \times 10^3\ M^{-1}$. From these values $k_2 = 0.385 \times 10^{-3}\ s^{-1}$ and $K = 6.66 \times 10^{-3}$ M can be calculated by the equation given in the Discussion. The rate of inactivation was low at pH values below 7 and considerably increased above pH 7.

If Dnp-sITP binds to the substrate binding site of the enzyme, then it is to be expected that ATP may protect the enzyme against the inactivation by Dnp-sITP. Alkaline pH values are favourable for the inactivation by Dnp-sITP. Alkaline pH values however, are unfavourable for ATP [31] and ADP binding [26]. In order to have optimal conditions for a protective action of ATP against the inactivation by Dnp-sITP the inactivation experiments were performed at pH 7.2. These experimental conditions result in a slower rate of inactivation (Fig. 8). Under these conditions, ATP protected the enzyme against the inactivation by Dnp-sITP. The specificity of the binding of Dnp-sITP at the ATP-binding site is demonstrated by the fact that Dnp-sIMP did not

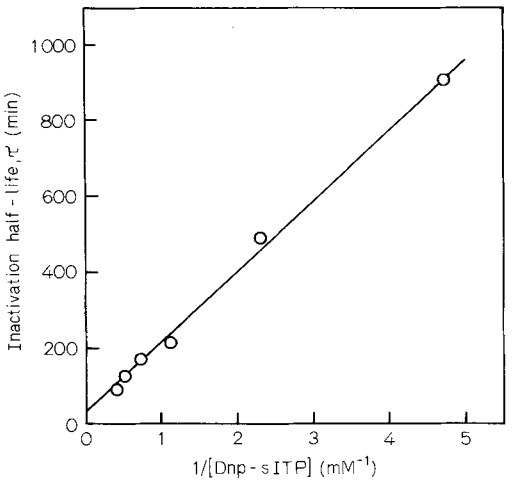


Fig. 7. *Inactivation half-life (τ) as a function of the reciprocal of the Dnp-sITP concentration.* The conditions were those of Fig. 6

inactivate the enzyme at the same concentrations as the corresponding triphosphate (Fig. 9).

Localization of the Inactivation by Dnp-sITP on Partial Reactions of $(Na^+ + K^+)$-Activated ATPase

In order to see which step of the overall reaction of $(Na^+ + K^+)$-activated ATPase is affected, the action of the ATP analogue on the Na^+-dependent protein phosphokinase reaction (Fig. 10) and the ADP-binding capacity of $(Na^+ + K^+)$-activated ATPase (Fig. 11) were studied. In agreement with the assumption of a covalent binding of Dnp-sITP to the

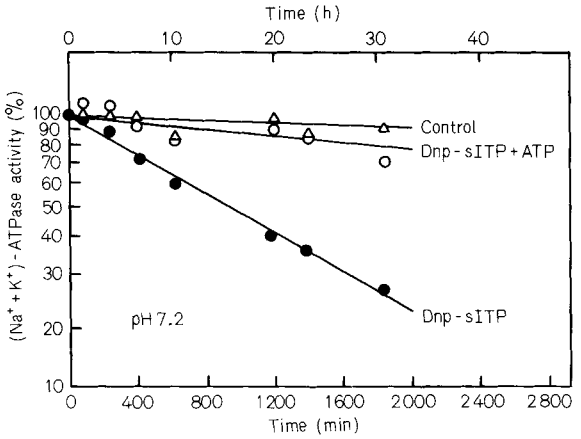


Fig. 8. *Protective effect of ATP against the inactivation by Dnp-sITP.* 1-ml reaction medium contained: 40 mM Tris-HCl pH 7.2, 0.64 mg protein of ($Na^+ + K^+$)-ATPase, 0.5 mM Dnp-sITP with or without 11 mM ATP. The control contained buffer only. Incubation temperature: 37 °C

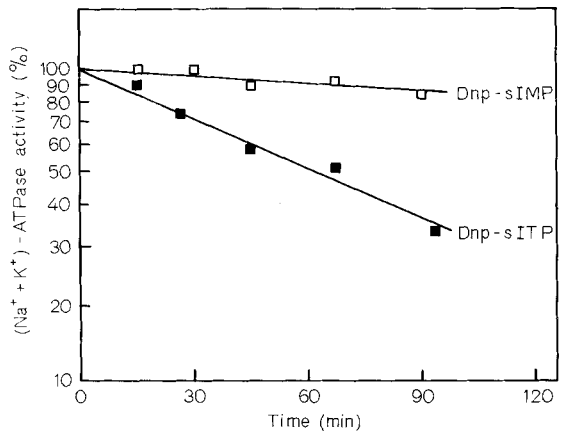


Fig. 9. *Comparison of the effects of Dnp-sIMP and Dnp-sITP on the inactivation of ($Na^+ + K^+$)-ATPase.* 2.1 mM Dnp-sIMP (□) or Dnp-sITP (■) was incubated at 37 °C with ($Na^+ + K^+$)-ATPase in 100 mM triethanolamine-HCl pH 7.9

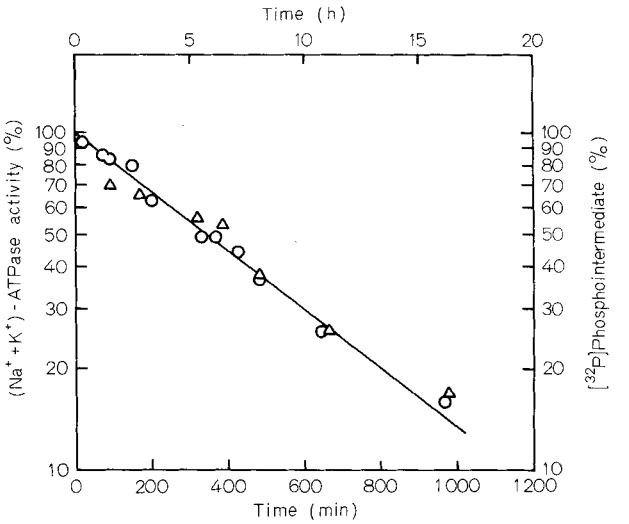


Fig. 10. *Effects of Dnp-sITP on ($Na^+ + K^+$)-ATPase activity and on the Na^+-dependent phosphoprotein kinase.* 64 mg protein of ($Na^+ + K^+$)-ATPase (spec. act. 2.85 U/mg) were incubated in a total volume of 25 ml with 40 mM Tris-HCl pH 8.2 and 0.79 mM Dnp-sITP at 37 °C. At the times indicated samples were withdrawn for activity determinations (○) and the measurements of phosphate incorporation (△). The difference at zero time between Na^+-dependent ^{32}P incorporation and the blank (containing 10 mM K^+ instead of 150 mM Na^+) was taken as 100%

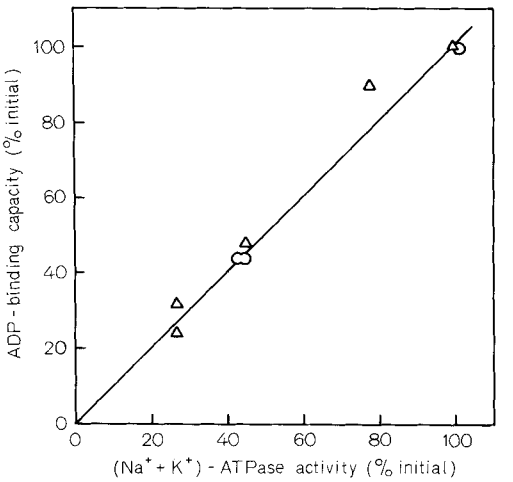


Fig. 11. *Effect of Dnp-sITP on the ADP-binding capacity and the activity of ($Na^+ + K^+$)-activated ATPase.* 120 mg of ($Na^+ + K^+$)-ATPase protein with a specific activity of 2.0 U/mg was inactivated in 41 ml total volume with 0.72 mM Dnp-sITP at pH 8.2 (Tris-HCl 100 mM). Samples of 10 ml were withdrawn and given to 1 ml 0.1 M reduced glutathione. ADP-binding capacity was determined after washing the enzyme with EDTA [26,27]. Circles and triangels represent different experiments

ATP binding site there was a decrease of the ability to form a phosphorylated intermediate in parallel with the decrease of the enzymic activity (Fig. 10). Moreover, the ADP-binding capacity decreased in a 1:1 relationship with the inactivation of the enzyme (Fig. 11).

Action of Dnp-sITP on K^+-Dependent Phosphatases

($Na^+ + K^+$)-ATPase preparations contain the activities of a K^+-dependent acetyl phosphatase and a K^+-dependent *p*-nitrophenylphosphatase [2]. Whilst the role of the K^+-dependent acetylphosphatase appears to be clearer, since it is hydrolyzed *via* the formation of a phosphointermediate which is identical

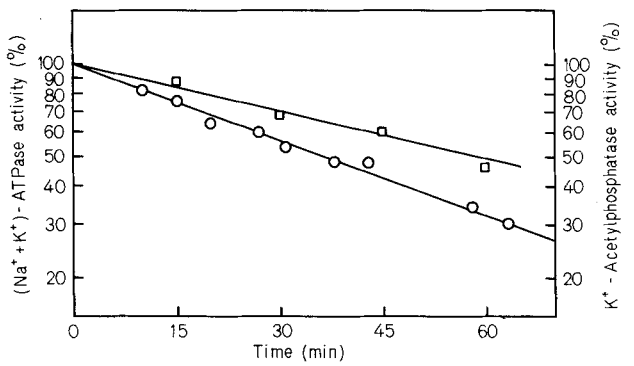


Fig. 12. *Comparison of the inactivation of $(Na^+ + K^+)$-ATPase (○) and K^+-activated acetylphosphatase (□).* 8.5 mg $(Na^+ + K^+)$-ATPase protein (spec. act. 3.0 U/mg) were incubated in 3.3 ml total volume at 37 °C with 20 mM Tris-HCl pH 8.2 and 1 mM Dnp-sITP

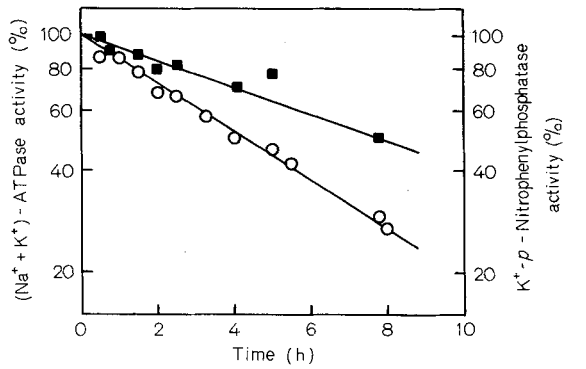


Fig. 13. *Comparison of the inactivation of $(Na^+ + K^+)$-ATPase (○) and K^+-activated* p-*nitrophenylphosphatase (■) by Dnp-sITP.* For experimental conditions see Methods and Fig. 11

with that formed from ATP [37], the role of the K^+-dependent *p*-nitrophenylphosphatase is less clear.

It is possible that the K^+-dependent *p*-nitrophenylphosphatase may reflect the K^+-dependent protein-phosphatase activity of the $(Na^+ + K^+)$-ATPase [Eqn (3)] [38,39], which is a step of the reaction sequence of the enzyme. Since —SH-binding reagents may act in a different way on the K^+-dependent acetyl phosphatase, *p*-nitrophenylphosphatase and the $(Na^+ + K^+)$-activated ATPase [40—42], we were interested to see whether this difference is also observable during the inactivation by Dnp-sITP. Fig. 12 and 13 demonstrate that the activities of the K^+-dependent acetylphosphatase and the K^+-dependent *p*-nitrophenylphosphatase decrease less rapidly than the activity of $(Na^+ + K^+)$-activated ATPase.

Incubation of $(Na^+ + K^+)$-Activated ATPase with clITP and sITP

Incubation of $(Na^+ + K^+)$-activated ATPase with clITP did not inactivate the enzyme in contrast to those studies reporting a rapid inactivation of GMP reductase [11] and IMP dehydrogenase by clIMP [12] by the formation of a thioether with the enzymes. sITP however, inactivated the enzyme, whereas the monophosphate at the same concentration was without effect (Fig. 14).

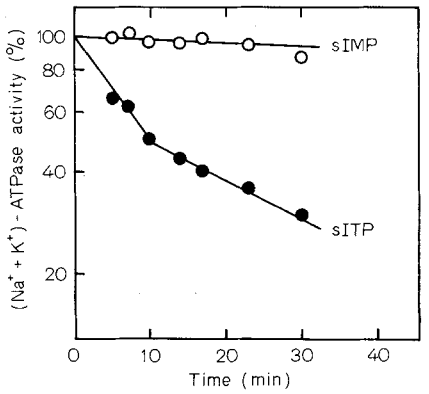


Fig. 14. *Comparison of the effects of sIMP and sITP on $(Na^+ + K^+)$-ATPase.* 0.6 mg protein of $(Na^+ + K^+)$-ATPase (spec. act. 2.1 U/mg) was incubated with 100 mM triethanolamine HCl pH 7.9 and 6 mM nucleotide at 37 °C

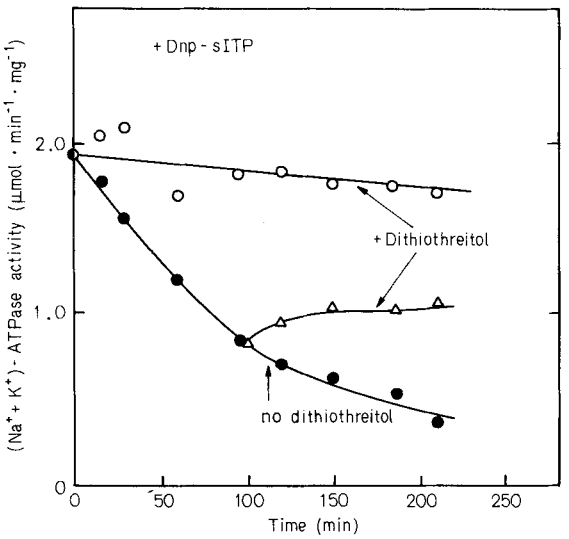


Fig. 15. *Effect of dithiothreitol on the inactivation of $(Na^+ + K^+)$-ATPase by 1.24 mM Dnp-sITP.* The enzyme was incubated in 100 mM triethanolamine pH 7.9 at 37 °C with (○) or without (●) 2 mM dithiothreitol (total volume 1 ml). At the time indicated 2 µmol dithiothreitol were added to the mixture containing no dithiothreitol (△)

Effects of Dithiothreitol on the Inactivation of $(Na^+ + K^+)$-ATPase by Dnp-sITP and sITP

According to Faust *et al.* [14], Dnp-sITP forms a thioether bond with proteins. In contrast to this analogue, sITP is assumed to form a mixed disulfide

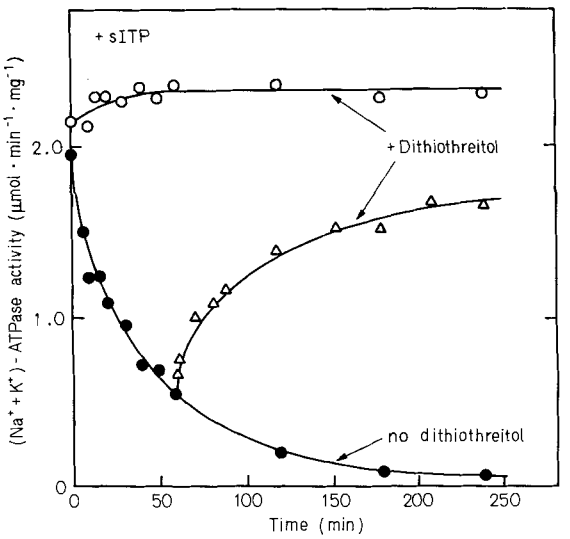


Fig. 16. *Effect of dithiothreitol on the inactivation of* $(Na^+ + K^+)$*-ATPase by 6 mM sITP.* For details see Fig. 15 and Methods

with proteins [13]. If this were correct dithiothreitol should hinder the inactivation by both ATP analogues. Fig. 15 and 16 demonstrate that both ATP analogues do not inactivate the enzyme in the presence of dithiothreitol. Moreover it is evident that dithiothreitol blocks the inactivation process by Dnp-sITP (Fig. 15) whereas the enzymic activity is partly restored if the enzyme was previously inactivated by sITP (Fig. 16).

DISCUSSION

Dnp-sITP and sITP very probably inactivate $(Na^+ + K^+)$-ATPase by binding to the ATP-binding site of the enzyme. This conclusion is based on the following findings.

a) Dnp-sITP and sITP inactivated the enzyme but Dnp-sIMP and sIMP did not (Fig. 6, 9, 14).

b) ATP protected $(Na^+ + K^+)$-ATPase against the inactivation by Dnp-sITP (Fig. 8).

c) Dnp-sITP treatment decreased the number of ATP-binding sites and the amount of Na^+-dependent phosphointermediate in parallel with $(Na^+ + K^+)$-ATPase activity (Fig. 10, 11).

d) sITP and Dnp-sITP have substrate functions for $(Na^+ + K^+)$-ATPase (Fig. 4A, 5).

e) The inactivation velocity of $(Na^+ + K^+)$-ATPase by the ATP analogues reached limiting values at high inhibitor concentrations (Fig. 7).

This saturation effect [44, 45] confirms that the inactivation by Dnp-sITP is preceded by a reversible binding to the enzyme [Eqn (5)]

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E - I\,(\text{inactive}) \qquad (5)$$

and is not simply random bimolecular. According to Gold and Fahrney [45] $K = (k_{-1} + k_2)/k_1$ [Eqn (5)] and the first-order rate constant for the actual inactivation step k_2 can be calculated for such two-stage inactivations from the kinetic expression [Eqn (6)]

$$\frac{1}{k} = \frac{1}{k_2} + \frac{K}{k\,[I]} \qquad (6)$$

where k is the observed first-order rate constant for inactivation and [I] is the inhibitor concentration. If k_{-1} is much greater than k_2, then K closely approximates to the inhibitor-enzyme dissociation constant. For Dnp-sITP at pH 8.2 and 30 °C an apparent first-order rate constant k_2 for inactivation of 0.385×10^{-3} s^{-1} was calculated. This is considerably lower than the k_2 value of 0.125 s^{-1} found for the inactivation of the inosine-5'-phosphate dehydrogenase by clIMP [12] and still 1/10 lower than other affinity labels [44, 46]. The apparent dissociation constant of the Dnp-sITP · enzyme complex at pH 8.2 and 30 °C was 6.66×10^{-3} M. This value is 1331-fold higher than that determined at 4 °C and pH 7.25 from [^{14}C]ADP competition experiments (Table). The discrepancy between these two determinations can be explained by the different experimental conditions: The dissociation constant of the ATP · enzyme complex at 0 °C increases sharply above pH 7.5 (Fig. 9 in [31]). In addition higher temperatures should increase the dissociation constant of the ATP · enzyme complex. Nevertheless it would be desirable to find an ATP affinity label with higher affinity for the ATP-binding site of $(Na^+ + K^+)$-ATPase.

Inhibition of $(Na^+ + K^+)$-ATPase by Dnp-sITP and sITP could be blocked by dithiothreitol (Fig. 15, 16). The sITP-inactivated enzyme regained its activity in the presence of dithiothreitol (Fig. 16) but enzyme which had been pretreated with Dnp-sITP did not (Fig. 15). These findings suggest that both ATP analogues inactivate $(Na^+ + K^+)$-activated ATPase by reacting with a sulfhydryl group in the ATP-binding site. In analogy to the reaction mechanism already described by Faust *et al.* [14], it seems that Dnp-sITP is forming a new thioether attached to the 6 position of the purine moiety with $(Na^+ + K^+)$-ATPase. sITP probably forms a disulfide with a cysteine residue of the enzyme [13]. The demonstration of an inactivation of Na^+-dependent phosphorylation reaction by Nbs_2 in parallel with $(Na^+ + K^+)$-ATPase activity (Fig. 1A) and the protective effects of ATP and ADP against the inactivation (Fig. 2) also agree with the assumption of a sulfhydryl group in the ATP-binding site. Since the 6-amino group of the purine ring is most essential for a high affinity binding of nucleotide triphosphates to the enzyme [31, 32] (Fig. 3) and since

ATP is the substrate with the highest hydrolytic rate for $(Na^+ + K^+)$-ATPase, it seems possible that the 6-amino group of the purine moiety of ATP forms a hydrogen bond with a cysteine in the ATP-binding site. This kind of interaction has been proposed by Barany and Merrifield [47] to occur with the ATP-binding protein of myosin.

Dnp-sITP also affected the associated reactions of the K^+-dependent acetylphosphatase and *p*-nitrophenylphosphatase (Fig. 12, 13). However, the inactivation of these enzymes was slower than that of $(Na^+ + K^+)$-ATPase. Acetylphosphate is probably hydrolysed at the ATP-binding site of $(Na^+ + K^+)$-ATPase, since acetylphosphate forms the same phosphointermediate as ATP [37] and it is a competitive inhibitor of $(Na^+ + K^+)$-activated ATP hydrolysis [53]. The relationship between $(Na^+ + K^+)$-activated ATPase and K^+-activated *p*-nitrophenylphosphatase is less clear. The enzyme may reflect the terminal step of the reaction sequence [Eqn (3)] [39]. The slower inactivation rate of the K^+-dependent phosphatases than $(Na^+ + K^+)$-ATPase may be explained in two ways.

a) It seems possible that acetylphosphate and *p*-nitrophenylphosphate are bound by the part of the ATP-binding site interacting with the triphosphate moiety of ATP. A covalent binding of the purine moiety to the enzyme may allow the dissociation of the triphosphate part of ATP from the substrate-binding site in the presence of K^+. Such a process could disclose this part of the active site for the binding of acetylphosphate and *p*-nitrophenylphosphate. It seems possible that this mechanism could result in a slower decrease of the activities of K^+-dependent phosphatases than of $(Na^+ + K^+)$-ATPase.

b) The phenomenon of the different inactivation rates of $(Na^+ + K^+)$-ATPase and K^+-dependent phosphatases could also be explained by the assumption of more than one catalytic center for K^+-dependent phosphatases per high-affinity ATP-binding site. Recently Jørgensen found in highly purified $(Na^+ + K^+)$-ATPase preparations two phosphorylation sites per ATP-binding and ouabain-binding site [48]. This finding suggests half-of-the-site reactivity of $(Na^+ + K^+)$-ATPase [49]. Two phosphatase activities have also been suggested by Skou [50].

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