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*INHIBITION BY DIISOPROPYLFLUOROPHOSPHATE OF A KIDNEY TRANSPORT ADENOSINE TRIPHOSPHATASE BY PHOSPHORYLATION OF A SERINE RESIDUE**

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In recent years, following initial observations of Skou,[1] there have been numerous studies of an ATPase[2] which is present in membrane preparations and which shows many of the characteristics of the coupled $Na^+ - K^+$ pumps; i.e., activation by the simultaneous presence of appropriate concentrations of Na^+ and K^+ and prevention of this activation by cardiac glycosides such as ouabain (see review[3]). This ($Na^+ + K^+$) activated, ouabain-inhibitable ATPase is often referred to as transport ATPase. The detailed mechanism of this transport ATPase is largely unknown, although there are indications that phosphorylation of an enzyme may be involved as an intermediate step.[4] This study is an attempt to gain more information about the transport ATPase.

DFP irreversibly inhibits a number of enzymes by phosphorylation of a serine residue at the active site.[5] Other serine residues on the same enzyme are unaffected. In this report it is shown that the transport ATPase in kidney membrane fragments is selectively and irreversibly inhibited by DFP. This inhibition requires Mg^{++}, is antagonized by Ca^{++}, and is prevented by low concentrations of ATP. The nontransport ATPase is unaffected by DFP. The ATPase preparation becomes labeled with P^{32} on incubation with DFP^{32} and Mg^{++}. Addition of protective concentrations of ATP reduces the labeling to less than half. Radioactive phosphoserine is recoverable from acid hydrolysates of the labeled ATPase preparation, and the amount of radioactivity in phosphoserine is reduced if the preparation is labeled in the presence of protective concentrations of ATP. This indicates that the DFP inhibition of the transport ATPase involves reaction with a serine residue. The fact that the transport ATPase and the DFP inhibition both require Mg^{++} and are inhibited by Ca^{++}, and the fact that ATP protects against DFP inhibition suggest that the reactive serine is at the ATP substrate site.

Experimental.—A heavy membrane fraction was prepared from beef kidney by a large-scale adaptation of the method of Kinsolving *et al.*[6] It was further treated with 0.6 *M* NaI by a modification of the method of Nakao *et al.*[7] This procedure preferentially removes the nontransport ATPase. The residue remaining after NaI treatment was washed twice with 250 mM sucrose containing 2 mM ethylene diamine tetraacetate neutralized with *Tris* (sucrose-EDTA) and finally suspended in sucrose EDTA (0.5 ml of sucrose EDTA per gm of original tissue) and stored in a dry-ice chest. The enzyme was thawed at room temperature just before use, washed two addi-

tional times in sucrose EDTA, and finally suspended in 50 mM imidazole-HCl buffer, pH 7.1. Unless otherwise indicated, preincubation with DFP was carried out at 37°C for 30 min with 10 mM DFP. The preincubation vessels contained 125 mM imidazole-HCl, pH 7.1, and approximately 125–250 μg of protein in a final volume of 1.0 ml. Other additions were made as indicated in the tables and figures. After preincubation the vessels were washed twice with sucrose EDTA, and the residues were suspended in 2.0 ml of 100 mM imidazole-HCl, pH 7.1. ATPase activity was assayed by a slight modification of a previous method.[8] The assay tubes contained 0.5 ml of a medium which contained membrane suspension (about 25 μg of protein), 80 mM imidazole-HCl (pH 7.1), 2 mM $MgCl_2$, 2 mM Na_2ATP, 80 mM NaCl, and 16 mM KCl. Incubation was for 20 min at 37°C. All assays were carried out in duplicate with the addition of either 0.05 ml of water or 0.05 ml of 1 mM ouabain. Protein was measured by the method of Lowry *et al.*[9] The ATPase activity in the presence of 0.1 mM ouabain will be referred to as the nontransport ATPase, and the increment in activity on omitting ouabain will be referred to as the transport ATPase. With these preparations, the transport ATPase activity ranged from 20 to 35 μmoles/mg protein/hr, and the nontransport ATPase ranged from 5 to 10 μmoles/mg protein/hr.

The transport ATPase was labeled with DFP^{32} by incubating 10 mg of protein at 37°C for 2 hr with 100 mM imidazole-HCl (pH 7.1), 25 mM $MgCl_2$, and 3 mM DFP^{32} (obtained from New England Nuclear Corp., Boston, Mass.) in a final volume of 0.5 ml. Inhibition of the transport ATPase was over 90%. The incubation mixture was then dialyzed against three changes of 4 liters of 100 mM *Tris*-HCl (pH 7.4) for 3 days. There was no loss in radioactivity after the last change. Aliquots of the protein were then counted directly, other aliquots were hydrolyzed with carrier phosphoserine, and 0.33 mg of protein hydrolyzate was electrophoresed on paper.

Results.—Time course of DFP inhibition: Incubation of the membrane preparation with DFP consistently inhibited the transport ATPase but was never observed to affect the nontransport ATPase; with 3 mM DFP the inhibition was essentially complete in 120 min without the addition of KCl, and in 60–90 min with the addition of 7.5 mM KCl (Fig. 1). With 10 mM DFP and without KCl the inhibition was essentially complete in 30 min (see other tables and figures). After treatment with DFP, washing of the preparation six times with sucrose EDTA did not lead to any recovery of transport ATPase activity; the inhibition therefore appeared to be irreversible. This is in contrast to the reversible inhibition by cardioactive steroids; when a membrane preparation in which the transport ATPase had been completely inhibited with 0.1 mM strophanthidin was washed six times, there was essentially complete recovery of the transport ATPase.

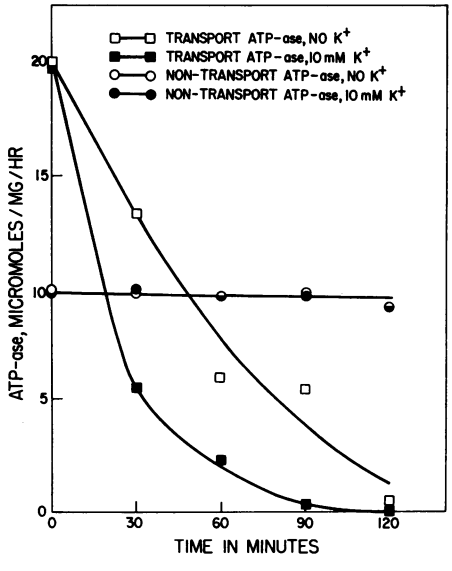

FIG. 1.—The time course of the inhibition of the transport ATPase by DFP. Preincubation was with 3 mM DFP for the indicated times. The preincubation vessels contained 24 mM $MgCl_2$, and buffer and enzyme as described under *Experimental*. The inhibition by DFP was stopped by adding 0.2 ml of 100 mM Na_2ATP.

Effects of Mg^{++} and Ca^{++}: The inhibition of the transport ATPase was completely dependent on the presence of Mg^{++}; half-maximal inhibition by DFP was obtained in the presence of about 0.3 mM $MgCl_2$, and the maximal inhibition was observed with about 3 mM $MgCl_2$ (Fig. 2). Concentrations of $MgCl_2$ up to

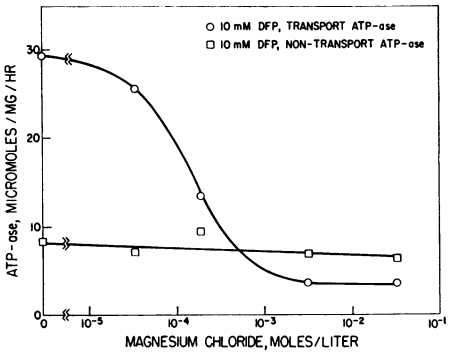

FIG. 2.—The effect of Mg^{++} on the inhibition of the transport ATPase by DFP.

25 mM had no significant effect if DFP was omitted. The inhibition of the transport ATPase by DFP was prevented by $CaCl_2$ (Fig. 3); 3 mM $CaCl_2$ led to half-maximal protection of the enzyme activity when 3 mM $MgCl_2$ was present. Higher concentrations of $CaCl_2$ completely prevented the DFP inhibition. There was some inhibition of the transport ATPase activity by Ca^{++} in the absence of DFP. Apparently Ca^{++} produced some change in the enzyme which was not reversed by two washings with sucrose EDTA. An inhibition of the transport ATPase when Ca^{++} is present during the assay has also been reported.[1, 10]

Effects of K^+ and Na^+: In view of known effects of Na^+ and K^+ on the transport ATPase, it was considered of interest to study the effect of these ions on the DFP inhibition. Twenty mM K^+ reduced the concentration of DFP for half-maximal inhibition to about one fifth (Fig. 4); 100 mM Na^+ was without significant effect on the DFP inhibition; 100 mM Na^+ partially reversed the K^+ effect.

Protection by ATP: Susceptible enzymes can often be protected against the inhibitory effects of DFP by addition of substrate.[5] In the absence of K^+, 6 mM ATP completely protected against the inhibitory effects of 10 mM DFP; the ATP concentration which afforded half-maximal protection was of the order of 0.1–0.5 mM (Fig. 5). This protective effect of ATP cannot be accounted for by chelation of Mg^{++}, since ATP protected in the presence of a marked excess of Mg^{++}. In the presence of 7.5 mM K^+, the concentration of ATP required for full protection was about 20 mM, and the concentration required for half-maximal protection about 15 mM (Fig. 5). One hundred mM Na^+ did not affect the ATP protection, nor did it significantly influence the antagonistic effect of K^+ on the ATP protection.

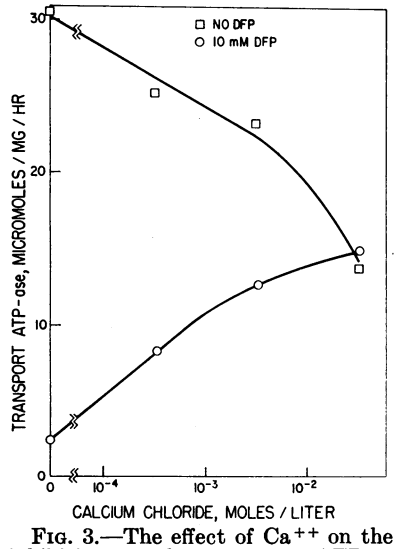

FIG. 3.—The effect of Ca^{++} on the inhibition of the transport ATPase by DFP. The concentration of $MgCl_2$ in the preincubation vessels was 3 mM.

CTP, GTP, ADP, pyrophosphate, and, to a slight extent, UTP protected the transport ATPase against inhibition by DFP, but the concentrations required for half-maximal protection were at least one order of magnitude higher than those for ATP (Fig. 6). AMP afforded no protection. These data suggest that a pyrophosphate group is necessary and sufficient for protection, but that the nature of the nucleoside group may enhance (e.g., adenosine) or antagonize (e.g., uridine) this protection. There was a slight ouabain-sensitive hydrolysis of ADP and CTP, but the hydrolysis of GTP, UTP, and pyrophosphate was not signifi-

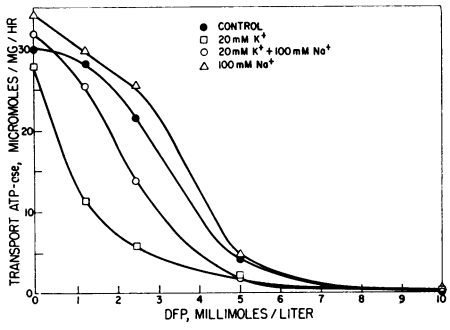

FIG. 4.—The inhibition of the transport ATPase at different DFP concentrations, and the effects of Na^+ and K^+ on this inhibition. The concentration of $MgCl_2$ in the preincubation vessels was 20 mM.

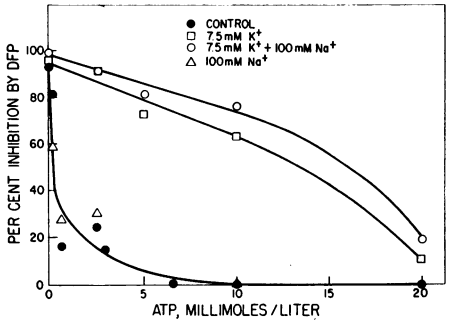

FIG. 5.—The protective effect of ATP on the inhibition of transport ATPase by DFP. The *Tris* salt of ATP was used. The concentration of $MgCl_2$ in the preincubation vessels was 24 mM. The per cent inhibition was determined by incubating vessels at each indicated ATP concentration without and with DFP.

cantly affected by ouabain (Table 1). The apparent ouabain-sensitive hydrolysis of ADP may have been due to myokinase activity in the preparations. These results would suggest that pyrophosphate and some of the nucleotides bind at the site and are hydrolyzed either not at all or very slowly. The possibility that the protection from DFP inhibition by CTP and the ouabain-sensitive hydrolysis of CTP were due to contaminating traces of ATP should be considered (the nucleotides were crystalline sodium salts obtained from Pabst Laboratories, Milwaukee, Wis.). Based on the Michaelis-Menten plot of transport ATPase versus ATP concentration, the concentration of ATP which would account for the ouabain-sensitive hydrolysis when 2 mM CTP was used as substrate would be about 0.002 mM ATP or 0.1 per cent contamination with ATP. This concentration of ATP would afford very much less protection against DFP inhibition than was observed with 2 mM CTP, so it can be concluded that the CTP protection was not due to contaminating ATP. Since pyrophosphate and GTP showed no significant ouabain-sensitive hydrolytic activity, it can be concluded that they also protected in their own right.

Effects of strophanthidin: Glynn,[11] as well as others, provided evidence that in the erythrocyte membrane cardioactive steroids compete with K^+ at the K^+ transport site (see, however, a recent note by Hoffman[12]). Glynn[11] suggested that since the five-membered lactone ring of the cardioactive glycosides is electrophilic, it may resemble K^+ in reacting with nucleophilic groups at the K^+ transport site. Figure 7 shows that strophanthidin acted like K^+, in that it lowered the concentration of

TABLE 1

OUABAIN-SENSITIVE HYDROLYSIS OF PYROPHOSPHATE AND VARIOUS NUCLEOTIDES

Substrate	μmoles Phosphate/mg Protein/hr		
	No ouabain	0.1 mM ouabain	Transport ATPase
ATP	44.7	8.5	36.2
GTP	11.5	10.6	1.9
CTP	8.2	5.9	2.3
ADP	11.0	5.6	5.4
UTP	8.1	8.0	0.1
PPi	6.6	6.5	0.1

Each substrate was 2 mM. Assay was carried out as described in text.

TABLE 2

PHOSPHORYLATION OF A SERINE RESIDUE IN THE TRANSPORT ATPASE PREPARATION BY DFP IN THE PRESENCE AND ABSENCE OF ATP

	Radioactivities in Fractions (cpm/mg Enzyme)				
	Total protein	Hydrolysate	Unknown	Phosphoserine	Inorganic phosphate
+ATP	9,200	6,900	308	2,670	5,520
−ATP	20,400	16,700	378	5,410	5,530
ATP-protected	11,200	9,800	70	2,740	10

The specific activity of the DFP was 165 μc/mg on the day of shipment. All radioactivities are corrected to this specific activity. Twenty μmoles of Na_2ATP was added at zero time and again at 60 min to the indicated vessel. The phosphoserine and the inorganic phosphate have been corrected for a 77% hydrolysis of standard phosphoserine run parallel with the protein samples. Other procedures are described in the text. The incomplete recovery of counts in the hydrolyzate is presumably due to insoluble protein remaining after hydrolysis.

DFP required for half-maximal inhibition of the transport ATPase. Under these conditions strophanthidin did not antagonize the effects of K^+ on the DFP inhibition.

Phosphorylation by DFP of a serine residue on the transport ATPase: DFP^{32} has been used to phosphorylate susceptible enzymes,[13] and radioactive serine phosphate has been isolated from acid hydrolyzates.[14] The transport ATPase preparation, which has so far resisted attempts at solubilization with preservation of activity, is impure, and treatment of such a preparation with DFP^{32} would be expected to phosphorylate more than one protein. However, the complete protection of the transport ATPase by its substrate suggested a possible means of specifically detecting labeling of the transport ATPase, since any increase in labeling on omitting protective concentrations of ATP would probably be due to labeling of the transport ATPase. If the enzyme preparation was incubated with DFP^{32} with and without ATP, there was a marked increase in labeling on omitting ATP (Table 2); this increment in labeling will be referred to as the ATP-protected labeling. Extraction of the protein with the solvent of Folch[15] removed no more than 10 per cent of the ATP-protected labeling. If the protein was treated for 20 min with 2 *N* HCl in a boiling waterbath and the liberation of P^{32}-inorganic phosphate was then determined,[16] about 10 per cent of the ATP-protected labeling was released as inorganic phosphate; on the other hand, heating in a boiling waterbath for 10 min with 0.5 *N* NaOH released about 45 per cent of the ATP-protected labeling as inorganic phosphate. The relative acid stability and alkali lability of the ATP-protected labeling suggested that the radioactivity might be due to a phosphoserine residue. Dialyzed enzyme preparations incubated with and without ATP were therefore hydrolyzed in 2 *N* HCl at 110°C for 10 hr with carrier phosphoserine, and the hydrolyzates were electrophoresed on paper as described previously.[16] A radioactive spot was seen on the autoradiogram which coincided with the carrier phosphoserine spot detected with either the phosphate stain of Wade and Morgan[17] or with ninhydrin. The intensity of the radioactive phosphoserine spot was less in samples derived from enzyme incubated with ATP. Similar results were obtained when the hydrolyzate was chromatographed in 0.8 *N* HCl in 70 per cent tertiary butanol[18] (R_F of phosphoserine, 0.38, and R_F of inorganic phosphate, 0.85). Quantitative measurement of the phosphoserine radioactivity as described previously[16] indicated that 28 per cent of the ATP-protected labeling of the hydrolyzate could be accounted for as phosphoserine-P^{32}. (This is based on a correction for 77 per cent hydrolysis of standard phosphoserine hydrolyzed under the same conditions.)

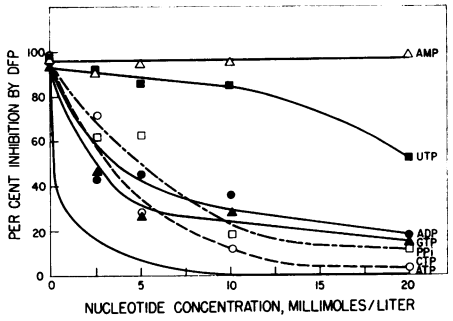

FIG. 6.—The effects of pyrophosphate and various nucleotides on the inhibition of transport ATPase by DFP. Pyrophosphate and the nucleotides other than ATP were added as the sodium salts. The concentration of $MgCl_2$ in the preincubation vessels was 24 mM. The per cent inhibition of transport ATPase was determined as in Fig. 5. The ATP protection curve from Fig. 5 is drawn in for comparative purposes.

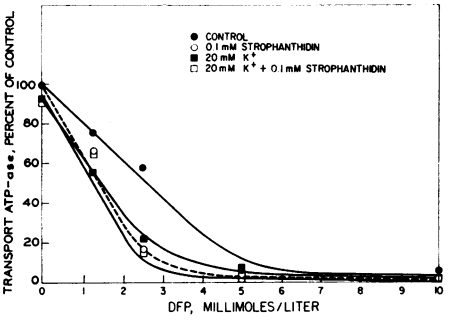

FIG. 7.—The effect of strophanthidin and K^+ on the DFP inhibition of the transport ATPase. The concentration of $MgCl_2$ in the preincubation vessels was 20 mM. Ten μl of 20 mM strophanthidin in dimethylformamide was added to the indicated vessels. Ten μl of dimethylformamide was added to all other vessels (this concentration of dimethylformamide had no significant effect on the transport ATPase). After preincubation, the enzyme was washed 3 times with sucrose EDTA before assay. The transport ATPase activities without and with strophanthidin but with no DFP were arbitrarily set at 100%.

There was a prominent radioactive inorganic phosphate spot on the electropherogram; 27–44 per cent of this could be accounted for as resulting from hydrolysis of phosphoserine. Most of the radioactivity in the inorganic phosphate spot must have been due to nondialyzable P^{32}-inorganic phosphate or material other than phosphoserine which liberated inorganic phosphate on hydrolysis. After correction for P^{32} resulting from hydrolysis of phosphoserine, there was no change in radioactivity in the inorganic phosphate on omitting ATP. There was also a faint radioactive spot below the phosphoserine spot, which showed less radioactivity in the ATP-protected sample. There were also very faint spots below this spot. These faint radioactive spots are probably peptides containing phosphoserine. The incomplete recovery of ATP-protected labeling as phosphoserine (after correction for hydrolysis of phosphoserine) is due, at least in part, to the fact that the protein was only partially hydrolyzed. Milstein and Sanger[19] recovered 15.6 per cent of the radioactivity as phosphoserine from P^{32}-labeled phosphoglucomutase hydrolyzed in 5.7 *N* HCl for 30 min at 100°C. Most of their remaining radioactivity was in the form of peptides. The above results show that there is an ATP-protected phosphorylation of a serine residue by diisopropylfluorophosphate; this serine residue is in all likelihood on a protein involved in transport ATPase activity.

Discussion.—Some ten enzymes have been shown to be inhibited by DFP.[5] With the exception of phosphoglucomutase,[19] these enzymes have been shown to be inhibited by phosphorylation of serine, presumably at or near the substrate site. The data presented here indicate that the transport ATPase system is inhibited by DFP, and this inhibition involves a phosphorylation of a serine residue of one or possibly more than one of the enzymes constituting this system. There are reasons to believe that this serine is at or near the substrate site. In the first place, inhibi-

tion of the transport ATPase by DFP can be completely protected by its substrate, as is the case for many other DFP-sensitive enzymes. In the second place, both the transport ATPase activity and the inhibition of the transport ATPase by DFP require Mg^{++} and are antagonized by Ca^{++}. This indicates that the conditions for DFP phosphorylation and the conditions for ATP binding and hydrolysis resemble each other closely. (Both may require the Mg-enzyme complex for binding.) These results can be most satisfactorily explained if one assumes that the ATP-binding site and the DFP-binding site is the same. A similarity in electronic distribution between pyrophosphate and fluorophosphate may be sufficient to permit binding of either at the ATP substrate site. The possibility that DFP could inactivate the enzyme by reacting at a site other than the substrate site cannot be absolutely ruled out. According to this line of reasoning, one mechanism of protection by ATP could be due to a conformational change produced in the enzyme when ATP binds at its substrate site, thus protecting the DFP-susceptible site which would be different from the substrate site. Alternatively, ATP may bind at two sites, one of them a nonsubstrate site, and this may be the site where the DFP reacts.

The fact that low concentrations of K^+ enhanced the inhibitory effect of DFP and antagonized the protective effect of ATP suggests that K^+ increases the relative affinity of DFP for binding to the enzyme. This effect is likely to be allosteric in nature, since the K^+ binds at the outside surface of the membrane[20, 21] and ATP binds at the inside surface. Since cardioactive steroids act at the outside surface of the membrane only,[22] presumably at the same site as K^+, it is perhaps not too surprising that strophanthidin acted like K^+ in lowering the concentration of DFP required for half-maximal inhibition of the transport ATPase. Na^+ did not appear to influence either the DFP inhibition or the ATP protection, but in high concentrations (100 mM) it did partially offset the potentiating effect of K^+ on the DFP inhibition; this is in keeping with its competitive effects on K^+ activation of the transport ATPase.[6]

It has been suggested, without supporting kinetic data, that a phosphoserine residue on a phosphoprotein may be an intermediate in the transport ATPase.[23, 24] Advocates of this hypothesis could argue that inhibition of the transport ATPase by phosphorylation of serine is evidence for phosphoserine as an intermediate in transport ATPase activity. However, in the cases of those enzymes which have been clearly shown to be inhibited by phosphorylation of serine by DFP, the mechanism of catalysis does not involve phosphorylation and dephosphorylation of the serine. In the case of phosphoglucomutase, which is the one instance of a DFP susceptible enzyme in which phosphorylation of serine is involved in the catalytic mechanism, Milstein and Sanger[19] could obtain no evidence for phosphorylation of the enzyme by DFP. If the recent studies of Charnock *et al.*[4] may be taken as evidence for the presence of a phosphorylated protein as an intermediate step in transport ATPase activity, then the site of phosphorylation by ATP is unlikely to be serine, since the protein-bound radioactivity which they measure is more acid-labile than phosphoserine. A more likely explanation of the data would seem to us to be that serine functions at the active site of an enzyme or enzymes involved in ATPase activity—presumably at the ATP substrate site—but that this serine does not itself undergo phosphorylation and dephosphorylation. Further investigation is required to settle this question.

Summary.—A transport ATPase preparation from beef kidney is irreversibly inhibited by DFP. This inhibition involves phosphorylation of a serine residue. The nontransport ATPase is unaffected by DFP. The inhibition requires Mg^{++} and is antagonized by Ca^{++}; these are known properties of the transport ATPase. Low concentrations of ATP protect against DFP inhibition. Pyrophosphate, CTP, GTP, ADP, and, to a slight extent, UTP also protect, but their affinities at the protecting site are at best only one tenth of that of ATP. AMP does not protect. The effects of pyrophosphate and the nucleotides suggest that a pyrophosphate group is necessary and sufficient for protection, but its affinity is influenced by the nature of the nucleoside group. K^+ enhances the inhibitory effect of DFP and antagonizes the ATP protection. Strophanthidin also enhances the inhibitory effect of DFP on the transport ATPase. Na^+ alone is without effect on either the DFP inhibition or the ATP protection, but it opposes the potentiating effects of K^+ on the DFP inhibition. The data suggest that a reactive serine is involved at or near the substrate site of the transport ATPase; however, these studies cannot be interpreted as evidence that this serine is phosphorylated by ATP during normal catalytic activity.

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† Research Career Awardee of the National Institutes of Health (GM-1347).

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[2] The following abbreviations are used. ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; DFP, diisopropylfluorophosphate; DFP^{32}, P^{32}-labeled diisopropylfluorophosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; ADP, adenosine diphosphate; UTP, uridine triphosphate; PPi, pyrophosphate; AMP, adenosine monophosphate.

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