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Interactions of a Photo-Affinity ATP Analog with Cation-Stimulated Adenosine Triphosphatases of Human Red Cell Membranes

(Na,K-ATPase/red cell ghosts/membrane probe)

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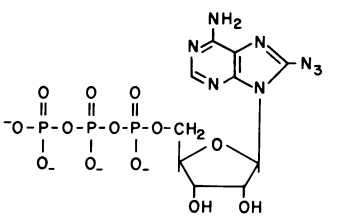
ABSTRACT To identify and isolate ATP binding and hydrolyzing sites of human red cell membranes we have synthesized a photo-activated ATP analog, 8-azido adenosine triphosphate (N_3ATP). In the absence of ultraviolet light it is a substrate for both the Mg-ATPase and the ouabain-sensitive, Na,K-ATPase. Hydrolysis of N_3ATP is prevented by increasing concentrations of ATP. Photolysis of N_3ATP with red cell membranes results in covalent incorporation and irreversible inhibition of both ATPase activities. Also, only three protein components of the red cell membranes are labeled. This labeling is completely abolished by appropriate concentrations of ATP.

Structural analogs of biologically active compounds have, in many instances, proven to be useful reagents or probes for studying biological processes. This is especially true of nucleotide analogs because of their major role in the control and regulation of cellular events. Following the pioneering work of Westheimer and colleagues (1–3), several investigators have recently used a variety of photo-affinity reagents to investigate the binding of various ligands to proteins (4–10). The advantages that photo-affinity reagents have over conventional reagents have been discussed by Knowles (11). These considerations indicated that a photo-affinity analog of ATP would be useful for isolating and identifying the ATP binding and/or hydrolyzing sites of various ATP utilizing systems. Therefore, a photo-activated ATP analog, 8-azido adenosine triphosphate



8-Azido Adenosine Triphosphate (N_3ATP)

has been synthesized and tested to determine its use as an enzyme and membrane structure probe. A brief account of this work has been previously presented (12).

MATERIALS AND METHODS

8-Azido-adenosine triphosphate (N_3ATP) was synthesized as follows: N_3AMP was prepared (80% yield) by heating 8-bromo-AMP (5 mmoles) at 75° in anhydrous dimethylformamide containing tri-*n*-octylamine-azide (10 mmoles). Pyrophosphate was coupled to N_3AMP, making N_3ATP (30% yield) by the Michelson method (13). Chromatographic separation of the reaction mixture was done on a DEAE-cellulose-HCO_3 column (60 cm × 2.5 cm) with a linear gradient of 3 liters of H_2O:3 liters of 0.5 M triethylammonium bicarbonate. N_3ATP was identified by (*1*) its photolytic activity, (*2*) the incorporation of ^{32}P from labeled pyrophosphate, (*3*) its R_f value and the R_f value of its enzymatic hydrolysis products and (*4*) its ultraviolet (UV) spectrum. Photolytic activity of the compounds was determined by their ability to bind irreversibly to cellulose on thin-layer plates (Eastman) when exposed to UV light (253.7 nm). Azido-adenine analogs after UV exposure would remain at the origin after ascending chromatography, whereas the adenine and bromo-adenine products would not. Additional identification of the compounds was obtained by thin-layer chromatography of products after their hydrolysis by alkaline phosphatase and snake venom phosphodiesterase. The 8-azido adenosine analogs of AMP, ADP, ATP, and cAMP all have similar UV spectra at pH 7.4 with a λ_{max} at 281. The molar extinction coefficient for the 8-azido adenine moiety is 1.33×10^4 (14). N_3ATP and N_3ADP are more sensitive to chemical breakdown than N_3AMP. This is probably due to the interaction of the phosphates with the azide.

Hemoglobin-free ghosts (erythrocyte membranes) were prepared from freshly drawn, heparinized, human blood and stored at −20° as previously described (15). On the day of use the ghosts were thawed, washed twice at 20° with 35 ml of a solution that contained 80 mM NaCl, 40 mM KCl, 0.5 mM ethyleneglycol bis(β-aminoethyl ether)*N,N'*-tetraacetate (EGTA), 2.5 mM $MgCl_2$, and 20 mM Tris·HCl, pH 7.4. Washing was done using 70 volumes of this solution to resuspend the ghosts, followed by centrifugation (for 10 min at 2500 × *g*) and then aspirating off the wash solution. These frozen-thawed ghosts are freely permeable to ATP.

Duck salt gland Na,K-ATPase was a gift from Dr. Thomas Smith. Snake venom phosphodiesterase was obtained from Calbiochem Co. Alkaline phosphatase and all nucleotides were obtained from Sigma Chemical Corp. Radioactive [^{32}P]P_i (orthophosphate) and [^{32}P]PP_i (pyrophosphate) were obtained from New England Nuclear Corp. All organic reagents were obtained from Aldrich Chemical Co. or the J. T. Baker Co.

Photolysis of N_3ATP with ghosts was done with an ITT germicidal A (G30TA) lamp at approximately 20 inches

Abbreviations: UV, ultraviolet; EGTA, ethyleneglycol bis(β-aminoethyl ether)*N-N'*-tetraacetate.

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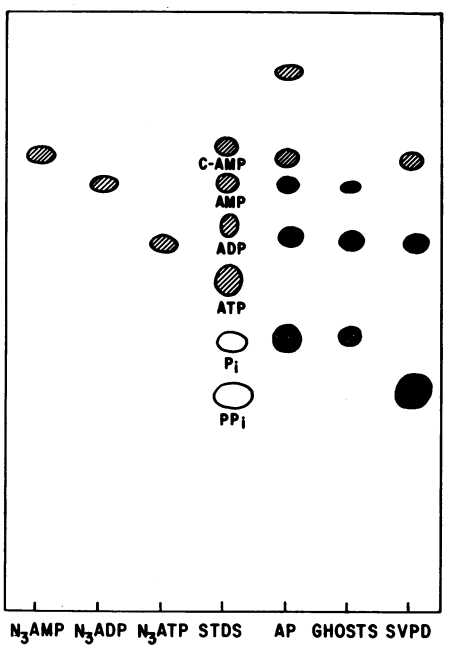

FIG. 1. Enzyme hydrolysis products of [β,γ-^{32}P]N_3ATP as determined by thin-layer chromatography. N_3ATP (4.5 mM) was incubated with 0.03 mg of alkaline phosphatase (AP) or 0.1 mg of snake venom phosphodiesterase (SVPD) at 25° in 0.2 ml of solution which also contained 20 mM Tris·HCl (pH 8.5) and 5 mM $MgCl_2$. The incubation with ghosts was done at 37° using the medium given in the legend of Fig. 2. Twenty-five-microliter aliquots were taken and spotted every 10 min for 2 hr. Chromatography was done using Eastman TL cellulose sheets with fluorescent indicator and a Gilman model 51325-1 chromatographic chamber. The solvent system was isobutyric acid:-NH_4OH:H_2O at 66:1:33 v/v. Cross-hatched and diagonal spots indicate location of radioactive and UV-absorbing spots, respectively. Completely dark spots indicate that both radioactivity and UV absorbance were present together. STDS is standards.

distance (energy output = 15 ergs/mm^2 per sec). The solutions being photolyzed (volume = 0.2–0.4 ml) were in glass serology plates which were kept cold by being partially immersed in an ice-water bath. Adenosine was added to maintain a relatively constant absorbance which prevented unequal inactivation due to unequal absorption of UV light by the ghosts. The solutions were mixed at 5-min intervals with an

TABLE 1. *Cation requirements for N_3ATP hydrolysis by red cell ghosts*

Additions			
15 mM K^+	30 mM Na^+	0.1 mM ouabain	nmoles of N_3ATP hydrolyzed
−	−	−	1.42
−	−	+	1.04
+	−	−	0.90
+	−	+	0.85
+	+	−	12.1
+	+	+	1.49

Assay conditions: Incubation was for 10 min at 37° in 1 ml of solution containing 0.5 mM N_3ATP, 20 mM Tris·HCl (pH 7.4), 1.05 mg of ghost protein, 0.5 mM EGTA, and 5 mM $MgCl_2$. Specific activity of [β,γ-^{32}P]N_3ATP was 669 cpm/nmole.

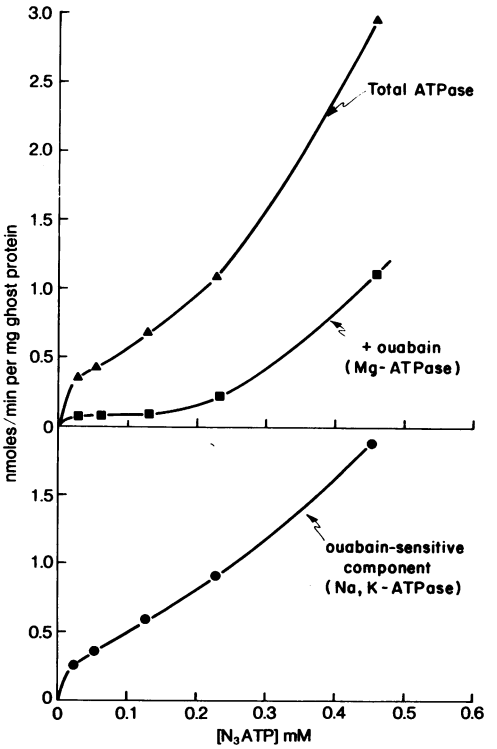

FIG. 2. Hydrolysis of N_3ATP by ATPases of red cell ghosts. Assay conditions: Ghosts were incubated for 10 min at 37° in 1 ml of solution which contained 20 mM Tris·HCl (pH 7.4), 30 mM NaCl, 15 mM KCl, 0.25 mM EGTA, and 1.25 mM $MgCl_2$ in the presence and absence of 0.1 mM ouabain. The ouabain-sensitive (Na,K-ATPase) activity was obtained by subtracting the Mg-ATPase activity (i.e., activity in the presence of ouabain) from the total ATPase activity and is plotted separately in the lower portion of the figure.

air stream. After photolysis the ghosts were washed at least twice with 10 ml of ATPase assay medium (see Fig. 2 *legend*) before being assayed for ATPase activity or prepared for gel electrophoresis. Gel electrophoresis and autoradiography were done as previously described (6,17). Hydrolysis of [^{32}P]P_i from [γ-^{32}P]ATP and [β,γ-^{32}P]N_3ATP was assayed by a charcoal extraction procedure (15).

RESULTS

Enzyme Hydrolysis Products of [β,γ-^{32}P]N_3ATP. Hydrolysis of [β,γ-^{32}P]N_3ATP by alkaline phosphatase gave four separate UV-absorbing spots that correspond to N_3ATP, N_3ADP, N_3AMP, and N_3-adenosine (Fig. 1). Autoradiography of thin-layer chromatography plates shows radiation corresponding to [^{32}P]P_i, [β,γ-^{32}P]N_3ATP, and [β-^{32}P]N_3ADP (Fig. 1). Hydrolysis of [β,γ-^{32}P]N_3ATP by snake venom phosphodiesterase gave two UV-absorbing spots corresponding to N_3ATP and N_3AMP. Autoradiography showed radiation migrated only with the original compound and in a non-UV-absorbing spot which migrated like PP_i (Fig. 1). Treatment of [β,γ-^{32}P]N_3ATP with ghosts and the Na,K-ATPase from duck salt gland gave two UV-absorbing spots which correspond to N_3ATP and N_3ADP and radioactive products with R_f values corresponding to [β-^{32}P]N_3ADP and [^{32}P]P_i.

Substrate Properties of N_3ATP. N_3ATP is a substrate for the Na,K-ATPase (ouabain-sensitive) and the Mg-ATPase (ouabain-insensitive) of human red cell ghosts. Fig. 2 shows

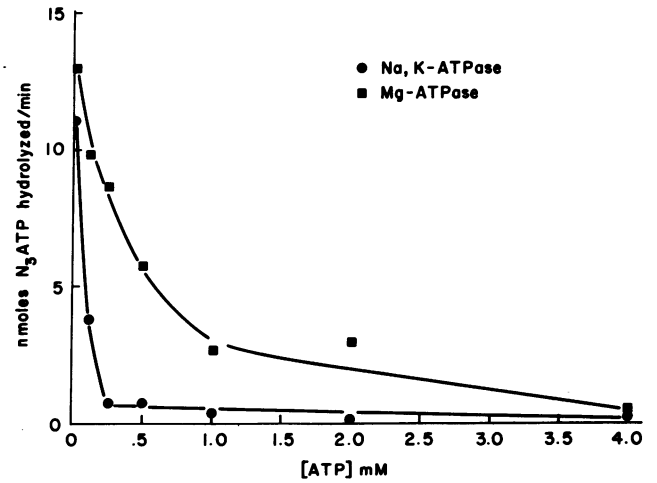

FIG. 3. Effect of [ATP] on the hydrolysis of N_3ATP by ATPases of red cell ghosts. Assay conditions were the same as for Fig. 2 except 2.1 mg of ghost protein were present. N_3ATP concentration was 0.225 mM (44,750 cpm/μmole).

the N_3ATP concentration effects on the rates of hydrolysis by the ouabain-sensitive and insensitive components. Table 1 shows the ouabain-sensitive hydrolysis also requires the presence of both Na and K. N_3ATP is also a substrate for the partially purified, ouabain-sensitive ATPase from duck salt gland. Our studies have shown that the apparent K_m of N_3ATP with the Na,K-ATPase from human red cell ghosts and duck salt gland is similar to that of ATP (approximately 0.1 mM). This value is in agreement with other published results, especially when one considers the effect that varying potassium concentration has on the apparent K_m of ATP (18).

Fig. 3 shows the effect of increasing ATP concentration on the hydrolysis of [β,γ-^{32}P]N_3ATP by the ouabain-sensitive and insensitive components of red cell ghosts. It is evident that ATP more effectively prevents hydrolysis of N_3ATP by the ouabain-sensitive component than by the insensitive component.

Inactivation of the ATPase Activities by Photolysis with N_3ATP. The Mg-ATPase and Na,K-ATPase activities are inhibited by UV light if treated as given in Table 2. Both adenosine and ATP protect against this inactivation, with ATP being the better protector at equal concentrations. This indicates that ATP protects by absorbing UV light while unbound, like adenosine, and in addition further protects the enzyme by binding to the active site. Photolysis in the presence of N_3ATP results in a marked inhibition of both ATPase activities at levels considerably below that obtained in the absence of this reagent. ATP protects the Na,K-ATPase from this inhibition to a much greater extent than it protects the Mg-ATPase (Table 2). This is to be expected since equal concentrations of ATP decrease hydrolysis of N_3ATP by the Na,K-ATPase to a greater degree than they decrease hydrolysis by the Mg-ATPase (Fig. 3). Therefore, ATP is more effective at keeping N_3ATP out of the Na,K-ATPase active site than it is at keeping it out of the Mg-ATPase active site. If this is true, then photolysis of ghosts in the presence of a 1:2 molar ratio of ATP:N_3ATP should result in greater inhibition of the Mg-ATPase at various nucleotide concentrations.

To attempt differential inhibition of the Mg-ATPase and Na,K-ATPase we photolyzed the red cell ghosts with a 1:2

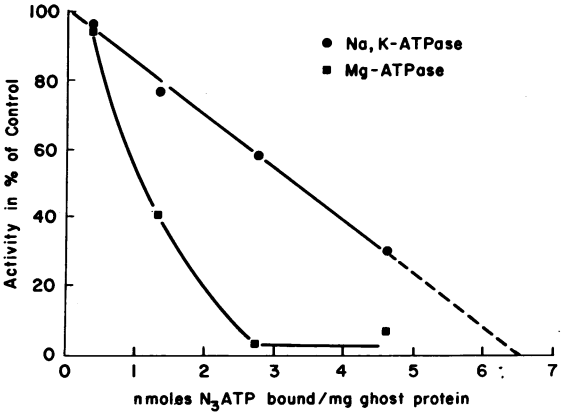

FIG. 4. Correlation of photo-catalyzed incorporation of N_3ATP onto red cell ghosts and ATPase activity. The photolyzed solution contained 10 mM Tris·HCl, pH 7.4, 2.5 mg of ghost protein, 40 mM NaCl, 20 mM KCl, 25 mM $MgCl_2$, and various amounts of ATP + N_3ATP in a 1:2 molar ratio. Adenosine was added to maintain an approximately constant solution absorbance. Photolysis was for 60 min at 0° as described in *Materials and Methods*. N_3ATP concentrations were 1.0, 2.4, 4.5, and 7.7 mM, respectively, giving decreased ATPase activity. The photolyzed red cell ghosts were washed twice with ATPase assay medium (10 ml) containing 1 mM ATP. In between each wash was a 10-min incubation to remove any ^{32}P that was enzymatically incorporated into the ghost protein. Assay medium (1.5 ml) was then added to the washed ghosts and equal portions (0.5 ml) were taken for ATPase assay ± ouabain. The final 0.5-ml fraction was precipitated and washed twice with 10 ml of 6% perchloric acid, 0°, containing 1 mM ATP. The perchloric acid precipitate was dissolved in 0.5 ml of Nuclear Chicago solubilizer and added to 10 ml of Bray's solution for measurement of radioactivity (16).

ratio of ATP to N_3ATP (as given in Fig. 4). In this experiment adenosine was added to keep the absorbance of the solutions approximately constant and high enough to prevent inactivation due to UV exposure within the time limits of the experiment. Sufficient Mg^{++} was added to bind all the nucleotide triphosphates present (other experiments not reported here have shown that Mg^{++} was necessary for N_3ATP hy-

TABLE 2. *Photo-dependent N_3ATP inhibition of red cell ghost ATPase activities*

Additions				% Inhibition	
[Adenosine] mM	[ATP] mM	[N_3ATP] mM	UV	Mg-ATPase	Na,K-ATPase
–	–	–	–	0	0
–	–	–	+	30	23
1.80	–	–	+	19	10
0.90	0.90	–	+	10	0
0.90	–	0.90	+	24	30
–	0.90	0.90	+	20	11
–	–	0.63	+	32	53
–	2.5	0.63	+	8	4
–	–	0.90	–	0	0

The ghosts were photolyzed for 60 min at 0° in a solution containing 20 mM Tris·HCl, pH 7.4, 30 mM NaCl, 15 mM KCl, 0.25 mM EGTA, and 5 mM $MgCl_2$. After photolysis the ghosts were washed twice with ATPase assay medium (see Fig. 2 legend) and assayed for ATPase activity as given in Fig. 2 except 0.4 mM ATP was used as the substrate.

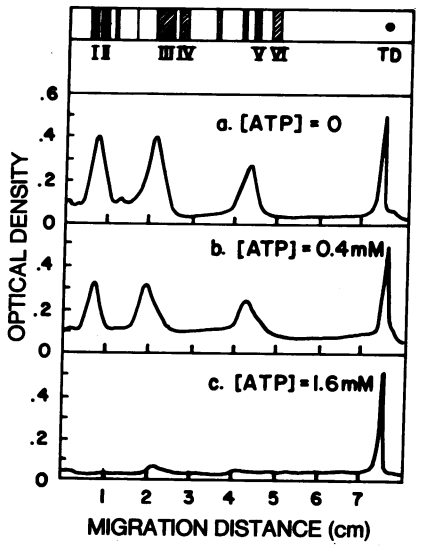

FIG. 5. Sodium dodecyl sulfate-acrylamide gel electrophoresis of ghosts labeled photocatalytically with [β,γ-^{32}P]N_3ATP. Procedure: Ghosts (0.25 mg) were photolyzed and treated as given in the legend of Fig. 4 with the exception that the N_3ATP concentration was 0.4 mM and the ATP concentration was 0, 0.4, and 1.6 mM, respectively, in (a), (b), and (C), respectively. The drawing at the top shows a gel (5.6%) on which the photolyzed ghosts proteins have been electrophoresed and stained with Coomassie blue. Photolysis with or without N_3ATP had no noticeable effect on the gel staining pattern of ghost protein when compared to a nonphotolyzed control. The bands are numbered as suggested by Fairbanks *et al.* (17). The graphs are densitometer traces of autoradiograms of gels on which photolyzed ghost proteins have been electrophoresed. The densitometer traces are roughly aligned with the drawing according to the position of the Pyronin Y marker, TD.

drolysis and optimum N_3ATP photo-catalyzed inhibition). Under these conditions the relationship between nmoles N_3ATP bound per mg of ghost protein and inhibition of Na,K-ATPase was found to be linear. The Mg-ATPase was inhibited to a greater degree than the Na,K-ATPase. This is the opposite result obtained in the absence of ATP but predictable, since ATP competes more effectively with N_3ATP for the active site of the Na,K-ATPase than it does for the active site of the Mg-ATPase (Fig. 3). To insure that the radioactivity incorporated was due to photo-catalyzed binding and not to enzymatic phosphorylation, the membranes were treated with nonradioactive ATP as given in the legend of Fig. 4.

In a control with [β,γ-^{32}P]N_3ATP, but without photolysis, less than 2% of ^{32}P was incorporated when compared to an identical photolyzed system. In another experiment the amount of ^{32}P incorporation was shown to be linearly dependent on the length of time of photolysis. Photolysis caused no inhibition of ATPase activity if N_3ATP was replaced by ATP (under conditions given for Fig. 4). These results indicate that ATPase inhibition and ^{32}P incorporation, from [β,γ-^{32}P]-N_3ATP is the result of covalent binding through a nitrene intermediate.

Covalent Labeling of Red Cell Ghosts with N_3ATP. The exposure of red cell ghosts to UV light in the presence of [β,γ-^{32}P]N_3ATP results in the covalent incorporation of label (Table 3). These results show that most of the ^{32}P incorporation requires UV light and is decreased by 40% when ATP is

TABLE 3. *Photo-activated incorporation of [^{32}P]N_3ATP into red cell ghosts*

Conditions	^{32}P Incorporated (cpm)
Control	0
N_3ATP	0
N_3ATP + ATP	433
N_3ATP + UV light	4,500
N_3ATP + ATP + UV light	2,690

Procedure: The photolysis system contained 10 mM Tris·HCl, pH 7.4, 3.87 mg of ghost protein, 40 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM N_3ATP, and 1.0 mM ATP. Photolysis was for 60 min at 0°. Then the ghosts were washed three times with 20 mM Tris·HCl, pH 7.4, 1 mM ATP followed by two washes with a 6% perchloric acid, 0.1 mM ATP solution at 0°. The precipitated protein was dissolved in 0.5 ml of Nuclear Chicago solubilizer and its radioactivity measured in Fluor-toluene. Specific activity of N_3ATP = 467 cpm/nmole.

present. No radioactivity is incorporated onto ghosts when N_3ATP is previously photolyzed before addition of the ghosts. Experiments using autoradiography of sodium dodecyl sulfate–polyacrylamide gels on which ghosts labeled with N_3ATP have been electrophoresed indicate that most of the label is incorporated into three regions. These regions coincide with bands II, III, and V [using the nomenclature of Fairbanks *et al.* (17)]. ATP, at approximately four times the N_3ATP concentration, decreased incorporation into these bands below the level detectable by autoradiography (Fig. 5).

It is not surprising that the proteins in bands II and III were labeled by N_3ATP, since these bands are known to contain components that are phosphorylated with [γ-^{32}P]ATP (16, 19–22). Band II is also thought to be the divalent (Mg or Ca) metal ion ATPase (23). Band III is known to contain the protein whose phosphorylation is specifically stimulated by Na and Mg (16). It also contains the major glycoprotein of red cells and perhaps other proteins as well.

The protein in band V has a molecular weight of approximately 41,300 and is one of the ghost proteins eluted by low ionic strength washes (17). Its identity is not known to us at this time.

DISCUSSION

The results in this paper show that a photo-affinity analog of ATP has been synthesized which (*a*) is a substrate for the Na,K- and Mg-ATPases in the absence of UV light and (*b*) irreversibly inhibits these ATPases by forming a covalent bond on photolysis with UV light. [β,γ-^{32}P]N_3ATP was used in the labeling experiments because it is the radioactively labeled species most easily made in large amounts with high specific activity. The use of this compound raises the possibility that ^{32}P incorporation was due to enzymatic phosphorylation of proteins and not due to photo-catalyzed binding through a nitrene intermediate. However, the following data indicate that the ^{32}P incorporation is not due to an enzyme mechanism: (*1*) photolysis was required for label incorporation, (*2*) photolysis of N_3ATP before addition of membranes decreased label incorporation over 95%, (*3*) label could not be removed by repeated incubations with unlabeled ATP which would remove ^{32}P incorporated enzymatically and, (*4*) label incorporation increased linearly with increased time of photolysis. Other experiments, not discussed here, using both

^{14}C base-labeled and α-^{32}P-labeled nucleotide show radioactivity incorporated into proteins only when exposed to activating light. This indicates that photolysis causes a covalent binding via the purine ring and, therefore, that this binding is probably effected through a nitrene intermediate.

The data presented in Fig. 4 show a linear relationship between N_3ATP incorporation and inhibition of Na,K-ATPase activity. We do not believe that this should be interpreted as a measure of the ATP hydrolyzing sites per milligram ghost protein (at 100% inhibition at least 10^6 sites per ghost would be labeled), even considering the possibility of labeling several ATPases. For determination of the exact number of sites labeled the radioactivity should be located on the adenine ring of N_3ATP, since the phosphates of this compound are relatively labile. The large amount of N_3ATP bound is not surprising in view of the fact that others have reported large quantities of ATP (6×10^5 molecules per ghost at 1 mM free ATP) reversibly bound to red cell ghosts (15, 24–26), some of which may be combined in membrane pools that have been proposed to exist in association with the Na-K pump (27, 28). The fact that Mg is required for correlation of percent labeling with percent inhibition, as well as for substrate hydrolysis, indicates that Mg-N_3ATP is interacting at the active site of the ATPases. Therefore, N_3ATP may also be used to label the active sites of various enzymes that require ATP. It should be noted that with each different enzyme system the degree of specific labeling will depend on the binding constant of N_3ATP and medium conditions (i.e., presence of protecting factors, pH, temperature, etc.) during photolysis. For example, with N_3ATP the degree of nonspecific labeling can be partially controlled by adding segments of the parent compound (i.e., adenosine, AMP, PP_i, etc.) to a concentration just below the point where they start to prevent interaction of N_3ATP with the active site. Using this procedure we have found that adenosine greatly reduces the amount of covalent incorporation of N_3ATP required to give a specific degree of inhibition of the ATPase activities. Scavengers, as reported by others, may also be used to decrease nonspecific labeling (9). However, the scavengers should not compete with the photoreagent for binding at the active site or preferentially bind the photo-reagent (as bovine-serum albumin does with N_3ATP and N_3ADP, unpublished results). In cases where the photoreagent binds reversibly with high affinity, e.g., N_3-cyclic-AMP, the need for protector molecules or scavengers is minimized (B. Haley, manuscript in preparation).

N_3ATP is not a substrate for seryl and leucyl-tRNA synthetases and RNA polymerase. With the synthetases N_3ATP and N_3AMP are competitive inhibitors which irreversibly bind to and inhibit the enzymes when photoactivated (R. M. Waterson, B. Haley, and W. Koenigsberg, unpublished results). N_3ATP is a substrate for myosin (P. Wagner and R. Yount, unpublished results) and luciferase. The different substrate properties of N_3ATP with these various enzymes may be due to the probable *syn* conformation of N_3ATP if it is similar to other 8-substituted adenosine analogs (29). This would make N_3ATP a substrate to ATPases that hydrolyze the *syn* conformation of ATP and a competitive inhibitor of enzymes that require ATP in the *anti*-conformation.

Finally, we suggest that 8-azide-adenine analogs (N_3ATP, N_3ADP, etc.) may be potent reagents for covalently labeling the nucleotide binding sites in which the analog is either a substrate, competitive inhibitor, allosteric effector, or other protein conformation regulator.

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