2'-O-Dansyl Analogs of ATP Bind with High Affinity to the Low Affinity ATP Site of Na⁺/K⁺-ATPase and Reveal the Interaction of Two ATP Sites during Catalysis*

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Na⁺/K⁺-transport through mammalian cell membranes by Na⁺/K⁺-ATPase (EC 3.6.1.37) needs the interaction of ATP sites with different binding affinities during catalysis: one with catalytic (high affinity site) and one with regulatory properties (low affinity site). To find affinity labels for the latter one, the effects of 2'-O-dansylated ATP analogs on Na⁺/K⁺-ATPase and its partial activities were analyzed. DANS-ATP (2'-O-(6-dimethylaminonaphthalenesulfonyl)adenosine 5'-triphosphate) inhibited noncompetitively at low ATP concentrations and competitively at high ATP concentrations the Na⁺/K⁺-activated hydrolysis of ATP under turnover conditions. It interacted preferentially with the low affinity ATP site as shown by its protective effect against the inactivation of Na⁺/K⁺-ATPase by Co(NH₃)₄ATP and Cr(H₂O)₄ATP. DANS-N₃-ATP, however, inactivated Na⁺/K⁺-ATPase. The initial velocity of inactivation shows a sigmoid concentration dependence that was converted to a hyperbola in the presence of ATP. DANS-N₃-ATP inhibited competitively the K⁺-activated hydrolysis of p-nitrophenyl phosphate in a fluorescein isothiocyanate-blocked enzyme but did not affect Na⁺-dependent phosphoenzyme formation from [γ³²P]ATP in a Co(NH₃)₄PO₄-blocked enzyme. These effects could be described by a Koshland-Némethy-Filmer model assuming two nucleotide binding sites in strong cooperation. Fitting all data to this model revealed that ATP was bound in a negative cooperative way with a $K_d = 0.1$ μM to the first site and a $K_d = 100–120$ μM to the second site of the enzyme containing already one ATP bound. The hydrolysis of ATP through a pathway with two ATP bound was 30 times faster than hydrolysis with one ATP bound. DANS-N₃-ATP bound in a positive cooperative way with a $K_d = 500 ± 100$ μM to the first site and a $K_d = 2.5 ± 0.5$ μM to the second site containing already one DANS-N₃-ATP bound. Therefore, DANS-N₃-ATP may be an useful affinity marker of the low affinity, regulatory ATP site.

The sodium pump of animal cell membranes converts the energy of ATP hydrolysis into an electrochemical gradient of sodium and potassium ions. The process of ion transport is intimately connected to oscillations of the enzyme protein of Na⁺/K⁺-ATPase (EC 3.6.1.37) between at least two different conformations called E₁ and E₂. Many of the events during cation transport can be described by the Albers-Post model (for an overview, see Ref. 1): Na⁺/K⁺-ATPase binds ATP with high affinity to the sodium exporting E₁-form (E₁ATP binding site) and is consequently phosphorylated. After the release of sodium at the outer cell side and the following dephosphorylation, the enzyme needs a second binding of ATP. Therefore, ATP binds with low affinity to the E₂-form (E₂ATP binding site) of Na⁺/K⁺-ATPase and enhances the rate-limiting step of deocclusion of potassium during import (2, 3). In contrast to expectations deriving from this single ATP site model with its subsequent formation of the ATP sites, use of substitution-inert MgATP complex analogs has led to the postulate of a coexistence (in time and at different places) of both ATP sites (4). The Rpeke-Schön-Stein model (5) attempts to explain such a situation by shifting the energy excess of the sodium-transporting subunit to the potassium-transporting subunit. Each subunit follows a whole Albers-Post cycle but 180° out of phase. The bicyclic model of Plesner, on the other hand, gets its power from two ATP binding sites whose partial activities (Na⁺/ATPase, K⁺-phosphatase) are lower than the overall reaction (Na⁺/K⁺-ATPase). A single subunit does not have to pass all of the intermediates of the Albers-Post circle, but the sum fulfills all steps required for a whole turnover (6). However, there is still a lot of discussion about the intermediates shared by the partial reactions and the overall reaction (7, 8).

Substitution-inert MgATP complex analogs like CrATP¹ or CoATP are helpful tools to dissect the overall Na⁺/K⁺-ATPase activity by specific modifications of either the E₁ATP site or the E₂ATP site (9, 10). The activities of the E₁ATP binding site (for example ATP/ADP exchange and “frontdoor phosphorylation”) are unaffected by the inactivation of the E₂ATP binding site by Co(NH₃)₄PO₄ (11). Similarly, CrAMP-PCP, which inactivates the E₁ATP site but is unable to phosphorylate it, does not affect the activities of the E₂ATP site, namely ⁸⁶Rb⁻/K⁺-activated phosphatase activity, and “backdoor phosphorylation” (12, 13). Although substitution-inert metal ATP complexes are on the one hand helpful tools to get information on basic prop-

¹ The abbreviations used are: CrATP, βγ bidentate complex of chromium(III) tetraaquadendronic acid 5'-triphosphate; CrAMP-PCP, βγ bidentate complex of chromium(III) tetraaquadendronic acid (βγ-methylene) diphosphate; CoATP, βγ bidentate complex of cobalt(III) tetramineadenosine 5'-triphosphate; Co(NH₃)₄PO₄, cobalt(III) tetraminephosphate; PCP, phosphocreatine; DANS-N₃-ATP, 2'-O-DANS-8-N₃-ATP, 2'-O-(6-dimethylaminonaphthalenesulfonyl)-8-azidoadenosine 5'-triphosphate; DANS-N₃-ATP, 2'-O-(6-dimethylaminonaphthalenesulfonyl)adenosine 5'-triphosphate; E₁, E₂ATP binding site, CoATP-sensitive site with high affinity for ATP, E₁, E₂ATP binding site, CoATP-sensitive site with low affinity for ATP; TNP-ATP, 2'-3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; FITC, fluorescein isothiocyanate.
erties of the coexisting ATP binding sites, on the other hand their suipicial properties and the low affinity to the E1ATP site hamper a study of the interaction of these sites during catalysis. The interactions of the two ATP sites during Na+/K+ transport would be much easier to study if fluorescent ATP analogs exist which are able to discriminate between E1ATP and E2ATP sites. So far only the fluorescent TNP-ATP is known which binds with high affinity to the E1ATP site but is not hydrolyzed there (14). Therefore, we studied whether DANS-ATP and DANS-N2-ATP are substrates of Na+/K+ -ATPase and are able to distinguish between E1 and E2 conformations. We became aware that both compounds are not hydrolyzed and bind with much higher affinity to the E1ATP than to the E2ATP binding site. This conclusion could be derived from studies of the protective effect of dansylated ATP analogs on the remaining Na+/K+ -ATPase activity after selective poisoning of any ATP site by CrATP or CoATP as well as by kinetic analysis of the inactivation process of the enzyme by DANS-ATP. This led to a kinetic model describing the overall Na+/K+ -supported ATP hydrolysis as an interaction of two coexisting nucleoside triphosphate binding sites which vary in their cooperativity depending on the nature of the substrate molecule bound.

MATERIALS AND METHODS

Chemicals—Dansylchloride was obtained from Fluka (Buchs, Switzerland), Sephadex LH-20 from Pharmacia (Uppsala, Sweden). Lab-Trol is a protein standard used in clinical chemical analysis and was delivered by Baxter Dude (Dudingen, Switzerland). All other chemicals were of the highest available purity. [γ-32P]ATP was obtained through Hartmann Analytic (Braunschweig, Germany).

Synthesis of ATP Analogs—Synthesis of dansylated ATP analogs was performed by a modification of the original procedure (15). The triethylammonium salts of ATP or 8-N3-ATP were dissolved in freshly distilled dimethylformamide. An equimolar amount of dansyl chloride in dimethylformamide was added, and the ester formation was allowed to proceed under stirring for 2 h at room temperature. For the removal of remaining impurities after evaporation, the precipitate was dissolved in 50% ethanol (80 cm) swollen in distilled water. The column was washed with distilled water. Individual fractions of a first peak absorbing at 355 nm and containing the dansylated ATP derivative were subjected to thin layer chromatography (Silicagel G-60F254, 10:6:3 n-butyl alcohol/water/acetic acid). The Rf values of the ATP derivatives were: ATP, 0.1; DANS-ATP, 0.3; dansylate, 0.6. The product was analyzed by infrared spectroscopy (azido 2160 nm), UV-visible spectroscopy (adenine 257 nm, azidoazene 280 nm), and fluorescence spectroscopy (dansyl residue excitation 325 nm, emission 501 nm). Synthesis of CrATP, Co(NH3)4PO4, and CoATP was performed by the aniline procedure of Cleland and co-workers (16, 17). Co(NH3)4PO4 was synthesized according to Siebert et al. (18).

Enzyme and Assays—Na+/K+ -ATPase (EC 3.6.1.37) with a specific activity of about 15 units/mg was isolated from pig kidney by a modification of the method of Jørgensen (19). One enzyme unit is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of ATP/min at 37 °C under the conditions of the coupled optical enzyme assay (20). Protein was measured by the procedure of Lowry et al. (21) using Lab-Trol as a standard.

Study of the Substrate Function of 2′-O-Dansylated ATP Derivatives—Hydrolysis of 2′-O-dansylated ATP or 8-N3-ATP by Na+/K+ -ATPase was studied at 37 °C in two ways. (i) An optical assay was used which contained instead of ATP, a 100 μm concentration of the dansylated ATP derivatives, and the release of the corresponding ADP was followed from the oxidation of NADH + H+. The assay system consisted of 60 mM imidazole/HCl, pH 7.25, 100 mM NaCl, 60 mM NH4Cl, 3 mM MgCl2, 0.4 mM phosphoenolpyruvate, 0.7 mM NADH + H+, 3 units of pyruvate kinase, and 6 units of lactate dehydrogenase. (ii) The release of inorganic phosphate was determined after incubation of the dansylated ATP derivatives with 0.3 unit of Na+/K+ -ATPase in 60 mM imidazole/HCl, pH 7.25, 100 mM NaCl, 60 mM NH4Cl, and 3 mM MgCl2 for 15 min. The effect of dansylated ATP derivatives (0–25 μM) on ATP hydrolysis by Na+/K+ -ATPase was tested in the optical assay (20) in the concentration range of 0.5–1,000 μM ATP. The amount of enzyme was 0.02 unit in each experiment.

Study of the Protective Effect of 2′-O-DANS-ATP Analogs against the Inactivation of Na+/K+ -ATPase by CrATP and CoATP—Na+/K+ -ATPase (1 unit) was incubated in a total volume of 250 μl at 37 °C in 60 mM imidazole/HCl, pH 7.25, increasing concentrations of CrATP (0–200 μM) or CoATP (0–1 mM), and DANS-ATP in the range of 0–50 μM. Aliquots of 25 μl of incubation mixture were transferred 0–90 min after the addition of the inactivating enzyme to a Sephadex LH-20 assay for Na+/K+ -ATPase. The inactivation rate constants were calculated from a plot of the logarithm of the remaining activity against the inactivation time. The affinities of the analogs were analyzed from the rate constants according to Piskacewics and Smith (22).

Inactivation of Na+/K+ -ATPase by 2′-O-DANS-8-N3-ATP—Na+/K+ -ATPase (2 units) was incubated in the dark at 37 °C in a total volume of 500 μl in 50 mM imidazole/HCl, pH 7.25, with increasing concentrations of 2′-O-DANS-8-N3-ATP (0–100 μM) and various ATP concentrations (0–1,000 μM). The velocity of the inactivation of Na+/K+ -ATPase was followed by transferring 20-μl aliquots of the incubation mixture at various time intervals to the optical assay system. The initial rates of inactivation by 2′-O-DANS-8-N3-ATP were determined from the first linear part of a plot of the remaining activity against time.

Study of the Effect of 2′-O-DANS-8-N3-ATP on the Activity of the E2 ATP Site—Na+/K+ -ATPase (4 units) was incubated overnight at 37 °C in a total volume of 750 μl overnight at 37 °C in 20 mM Tris/HCl, pH 7.5, 20 mM NaCl, and 200 μM CrAMP-PCP. After centrifugation and washing with 10 mM Tris/HCl, pH 7.5, the enzyme was resuspended in 40 μl of 40 mM Tris/HCl, pH 7.25. It was then incubated in a total volume of 500 μl with increasing concentrations (0–50 μM) of DANS-ATP. The reaction was stopped by the addition of 250 μl of ice-cold water, followed by centrifugation for 30 min at 100,000 × g in a Ti-50 rotor of the Beckman Spinco ultracentrifuge. The enzyme was resuspended, and Na+/K+ -dependent phosphorylation from [γ-32P]ATP was carried out as described by Buxbaum and Schoner (11).

Study of the Inactivating Effect of 2′-O-DANS-8-N3-ATP on the Activity of the E2 ATP Site—Na+/K+ -ATPase (2 units) was incubated in a total volume of 750 μl overnight at 37 °C in 20 mM Tris/HCl, pH 7.5, 20 mM NaCl, and 200 μM CrAMP-PCP. After centrifugation and washing with 10 mM Tris/HCl, pH 7.5, the enzyme was resuspended in 40 μl of 40 mM Tris/HCl, pH 7.5. It was then incubated with increasing concentrations (0–100 μM) of DANS-ATP. The enzyme was resuspended in 500 μl of ice-cold water. The protein was spun down by centrifugation at 100,000 × g for 30 min. The pellet was resuspended in 500 μl of buffer, and the centrifugation was repeated. Finally, the pellet was resuspended in 140 μl of 75 mM Tris/HCl, pH 7.5, 7.5 mM MgCl2, and 15 mM KCl and used for determinations of the activity of p-nitrophenylphosphatase as described by Hamer and Schoner (12).

Koshland-Némethy-Filmer Model of ATP Binding at Two Substrate Sites—A kinetic model was developed to fit experimental points of Na+/K+ -ATPase activity to a two-site competitive system (23). The model assumes that binding of a first substrate (S) or inhibitor (I) molecule to the enzyme (E) and forming ES (or EI) complexes alters the binding affinity of the second molecule, SEI, LES, lessening (in the case of a positive cooperativity, interaction factor smaller than one) or by decreasing (in the case of negative cooperativity, interaction factor greater than one) the affinity of the empty binding site. The binding of substrate (inhibitor) is sequential, but the sites are located at different places. The interaction factors a, b, and c describe alterations in the affinity of a second site due to substrate (inhibitor) occupancy at a first site. The factor a describes a change in affinity due to interaction of substrate binding sites when a second substrate is bound (SES); the factor c describes the interaction of sites when the inhibitor is bound exclusively (IEI). Interaction factor b reflects the interaction of sites when both, substrate and inhibitor are bound and SEI, LES hybrid complexes are formed. The factor z refers to changes in the rate constant of product forming k4 due to IES hydrolysis. The factor y refers to changes in the inactivation rate constant k5 due to IEI inactivation (24). In the model presented (Eq. 1) the initial velocity of inactivation v0 is obtained by multiplying all inactive intermediates (IE, IE, SE, IES, and IEI) with the inactivation rate constant k5 (Equation 2). The initial velocity of substrate hydrolysis v0 is obtained by multiplying all product forming complexes (ES, IE, IES, and SES) with the rate constant of product forming k4 (Equation 3). The relation of the sum of all enzyme intermediates. Maximal velocity Vmax of inactivation is reached when all of the enzyme is in the IEI complex and Vmax when all of the enzyme is in the SES complex (Vmax = 2 k5 k4). The hybrid complexes are recognized as noncatalytic because the data did not fit significantly better to other models. In a modification of this model the protective effect of a ligand (S) against the inactivation of Na+/K+ -
FIG. 1. General allosteric mechanism. Hydrolysis and inactivation are explained at two sites according to Ref. 23. The rate constant of product formation $k_P$ is influenced by a factor $z$ when $P$ is formed through an SES complex (Equation 1). The rate constant of inactivation $k_i$ is altered by a factor $y$ when the inactivation process passes through double-occupied complexes (Equation 2). The dissociation constants of the substrate complex $K_s$ and the inhibitory complex $K_i$ are altered by the interaction factors $a$, $b$, and $c$. The hybrid complexes $ES$ are regarded as noncatalytic because of an easier understanding and no significant change in the fitting process by use of the more complex model. Intermediates in the dashed white box are excluded for nonhydrolyzing ligands. Intermediates in the other boxes are eliminated for the inactivation by the metal-ATP analogs (CrATP, Equation 3: black; grey; CoATP, Equation 4: white/black). $E$, free enzyme; S, substrate; I, inhibitor; * different enzyme conformations.

ATPase was developed (24). It takes into consideration the inactivation by CrATP only at the high affinity ATP binding site (9) and the inactivation of CoATP only at the low affinity ATP binding site (10) (Fig. 1). The hybrid complexes of metal-ATP analogs and protecting ligand are regarded as inactive intermediates. CrATP is eventually able to bind at the low affinity ATP site. CoATP has been shown to bind at high affinity ATP site (32). These interactions, however, are fully reversible and do not inactivate the enzyme. The ligand is able to bind at both ATP binding sites but is not hydrolyzed. The meaning of the constants and evaluation of the equations (Equations 3 and 4) follow the above definitions.

Overall catalysis is

$$V = \frac{[S]}{K_s + [S]} + \frac{[S]}{K_a + [S]}$$

(Eq. 1)

Inactivation by DANS-N$_3$-ATP is

$$V_i = \frac{[I]}{K_i} - \frac{[I]}{K_i + [I]}$$

(Eq. 2)

Inactivation by CrATP is

$$V_i = \frac{[I]}{K_i} - \frac{[I]}{K_i + [I]}$$

(Eq. 3)

Inactivation by CoATP is

$$V_i = \frac{[I]}{K_i} - \frac{[I]}{K_i + [I]}$$

(Eq. 4)

RESULTS

Study of the Interaction of DANS-ATP with Na$^+$-K$^+$-ATPase—Contrary to our expectations, neither DANS-N$_3$-ATP nor DANS-ATP was a substrate of Na$^+$-K$^+$-ATPase. Substrate hydrolysis was neither detectable in the optical assay nor upon prolonged incubation of the enzyme with the ATP analogs and determination of the inorganic phosphate liberated (data not shown). DANS-ATP had also no inactivating effect on Na$^+$-K$^+$-ATPase activity even after several hours of incubation at 37 °C (data not shown). However, DANS-ATP inhibited under turnover conditions the overall Na$^+$-K$^+$-activated ATP hydrolysis in a noncompetitive way at low ATP concentrations and competitively at high ATP concentrations (Fig. 2). The kinetics could be explained by a fitting of the data according to a Koshland-Némethy-Filmer model (Fig. 1, Equation 1), resulting in the following parameters: $K_{(ATP)} = 0.3 \pm 0.1 \mu M$, $K_{(DANS-ATP)} = 100 \pm 10 \mu M$, $a = 0.2 \pm 0.1$, $b = 0.2 \pm 0.1$, $c = 0.005 \pm 0.002$, and $z = 15 \pm 5$. This would mean that according to the kinetic model the second ATP binds with $K_{(ATP)} = 113 \mu M$ to the enzyme and that the turnover with two ATP sites occupied is 30 times faster than with one ATP bound. To understand why DANS-ATP may act this way, the interference of this fluorescent ATP analog with the MgATP complex analogs CrATP or CoATP, which inactivate specifically Na$^+$-K$^+$-ATPase (4), was studied. CrATP has formerly been shown to inactivate the E$^i$ATP site (9) and CoATP the E$^s$ATP site (10) of the enzyme. When the E$^s$ATP site was inactivated by CoATP, DANS-ATP protected the enzyme effectively at low concentrations (Fig. 3A) and was less potent when the E$^s$ATP site was inactivated by CrATP (Fig. 3B). The data could be interpreted by fitting the data according to a modified model (Fig. 1). The following parameters are evaluated for CrATP (Equation 3) and CoATP (Equation 4): $K_{(CrATP)} = 35 \pm 5 \mu M$, $K_{(CoATP)} = 0.5 \pm 0.2 \mu M$, $K_{(DANS-ATP)} = 250 \pm 50 \mu M$, $a = 0.61 \pm 0.005$, $b = 300 \pm 100$, $c = 750 \pm 250$, and $y = 0.7 \pm 0.2$. Obviously, the first molecule of DANS-ATP binds with $K_y = 250 \mu M$ and the second molecule with $K_y = 2.5 \mu M$ to Na$^+$-K$^+$-ATPase.

Study of the Interaction of 2'-O-DANS-N$_3$-ATP with Na$^+$-K$^+$-ATPase—DANS-N$_3$-ATP inactivated Na$^+$-K$^+$-ATPase in the dark in a concentration-dependent way. The inactivation process by DANS-N$_3$-ATP could be described by a biphasic time course (Fig. 4A). To find out whether the enzyme activity may reactivate within a period of 5 min, which is necessary to perform the optical assay, 3 mM ATP was added after completion of the inactivation of the enzyme, and the activity was followed for additional 5 min. No change of the activity was seen under these conditions (data not shown). Additionally, we learned that DANS-N$_3$-ATP inactivates not only the overall reaction of Na$^+$-K$^+$-ATPase but also its partial activities, namely Na$^+$-dependent formation of a phosphointermediate...
are fixed at $K_d$ for typical experiments. By use of Equation 3 we obtained best fits to the sigmoid function of inactivation velocity of complex analogs (4). The sigmoid function of inactivation versus the concentration of DANS-N3-ATP revealed that the inactivation process is described by a biphasic time course. The experiments were done with two different preparations of enzyme and DANS-N3-ATP. The determination of enzyme activity was performed in triplicate. If not indicated, error bars are smaller than the symbols used.

**Panel A**, the initial velocity of inactivation of Na+/K+-ATPase was determined in the dark as a function of DANS-N3-ATP at various ATP concentrations (■, no ATP; ♦, 10 μM; ●, 1,000 μM) from the first linear part of panel A. The curves were drawn by fitting the experimental points to the two-site model (Equation 2). The Hill-coefficient changed from maximal $n_H = 2.3 ± 0.3$ without ATP to minimal $n_H = 0.9 ± 0.1$ in the presence of ATP. The points are the S.D. of three different measurements with three different enzyme and two different DANS-N3-ATP preparations. Best fits were obtained for $K_I(E) = 250 ± 50$ μM DANS-ATP and $K_I(E) = 35 ± 5$ μM CrATP (■, none; ○, 10 μM; ♦, 30 μM; □, 40 μM DANS-ATP).

(data not shown) and the activity of a $K^+$-dependent $p$-nitrophenylphosphatase (data not shown).

**Study of the Rate of Inactivation of Na+/K+-ATPase as a Function of the Concentration of 2'-O-DANS-8-N3-ATP**—A kinetic analysis of the initial inactivation rate as a function of the concentration of DANS-N3-ATP revealed that the inactivation process of Na+/K+-ATPase was not explicable by a simple hyperbolic absorption isotherm as is the case with MgATP complex analogs (4). The sigmoid function of inactivation velocity versus the concentration of DANS-N3-ATP indicated a cooperation of substrate binding sites (Fig. 4B). ATP protected the enzyme against the inactivation by DANS-N3-ATP. It converted the sigmoidal inactivation curve caused by DANS-N3-ATP to a hyperbolic one, and consequently the Hill coefficient changed from maximal $n_H = 2.3 ± 0.3$ to minimal $n_H = 0.9 ± 0.1$. This effect could be simulated by the two-site Koshland-Nemethy-Filmer model (Fig. 1, Equation 2) assuming positive cooperativity between the sites interacting with DANS-N3-ATP and negative cooperativity for ATP. Best fits are obtained by using $K_I = 500 ± 100$ μM DANS-N3-ATP, $b = 0.07 ± 0.04$, $c = 0.005 ± 0.003$, and $y = 50 ± 10$ when the parameters for ATP are fixed at $K_I = 0.3$ μM ATP and $a = 377$.

**Analysis of the Effect of 2'-O-Dansyl-8-N3-ATP on Partial Activities of Na+/K+-ATPase**—To understand in which way the ATP derivative DANS-N3-ATP may lead to the inactivation of Na+/K+-ATPase, we made use of ATP site-specific MgATP and MgPO4 complex analogs (11, 12). They fix a specific ATP binding site in its conformation (4) thereby eliminating allosteric effects between partial reactions otherwise occurring in the native enzyme. Therefore, one specific ATP site was blocked by use of the MgATP analogs, and the effect of DANS-N3-ATP on the remaining activity was studied. The Na+-dependent phosphorylation of enzyme protein from [γ-32P]ATP represents a catalytic activity of the $E_1$ enzyme form, whereas $K^+$-activated $p$-nitrophenylphosphatase is linked to the $E_2$ state (1). We also made use of the fact that blocking of the $E_1$ ATP site of the enzyme by FITC does not abolish the activity of $K^+$-activated $p$-nitrophenylphosphatase. However, FITC prevents the enzyme from forming a Na+-dependent phosphoenzyme by [γ-32P]ATP (25).

The effect of DANS-N3-ATP on the $E_1$ ATP site was analyzed by blocking the $E_1$ ATP site of Na+/K+-ATPase with Co(NH$_3$)$_2$PO$_4$ (11). Then the effect of the ATP analog on the residual activity of the Na+-dependent formation of a phosphointermediate was studied. To avoid a loss of the nomenclature label and to be sure of a complete blockade, we used long incubation times with Co(NH$_3$)$_2$PO$_4$. Thereby, some of the enzyme was poisoned by metal ions and was not able to get fully phosphorylated in relation to native enzyme. Nevertheless, we were able to show that on one hand the remaining capacity of 33 pmol of 32P/unit was accessible for 50 μM FITC. On the other hand, it was evident by the effect of up to 60 μM DANS-N3-ATP on phosphoenzyme formation that the Na+-dependent protein-phosphokinase reaction was not affected (Fig. 5A). These findings seem to indicate that DANS-N3-ATP either inactivates scarcely or binds fully reversibly to the $E_1$ ATP site.
the alteration of $K^+$-activated $p$-nitrophenylphosphatase in a CrAMP-PCP-blocked enzyme by DANS-N$_3$-ATP (Fig. 5C). The $K_I$ value for DANS-N$_3$-ATP calculated from this experiment by linear regression analysis was $10 \pm 3 \mu M$, but the $K_I$ value for the protective effect of ATP was $350 \pm 200 \mu M$. Thus, DANS-N$_3$-ATP interacts at the low affinity $E_2$ATP site with high affinity.

**DISCUSSION**

The function and location of the low affinity ATP binding site are still under discussion. There is much evidence that two ATP binding sites coexist in working $Na^+/K^+$-ATPase (4). It is unknown, however, if these sites are located on the same or on different catalytic subunits or if one subunit subsequently changes its behavior. Recently, Asakri’s group pointed out that coupling between the low and high affinity ATP sites is essential for the overall catalysis itself and not only for $K^+$ deoclusion. This group proposed also that the two ATP sites are distinct physical entities and that both conformational states may coexist (26). Additional evidence for the existence of two separated ATP binding sites was also obtained in phosphorylation experiments by extraphosphorylation from $p$-nitrophenyl phosphate (27) and by superphosphorylation from ATP (28). To explain their data, Peluffo et al. developed a model which allowed binding of up to three molecules of ATP to sites which exist in a “resting state” of the enzyme. This disappears as soon as the enzyme starts catalysis (29). Negative cooperativity of ATP hydrolysis in the overall $Na^+/K^+$-ATPase has been interpreted to indicate interaction of ATP sites within an oligomeric enzyme (5). However, negative cooperativity in ATP hydrolysis which does not require an oligomeric structure has been suggested from experiments on analytical ultracentrifugation of the solubilized $Na^+/K^+$-ATPase (30). Therefore, an equilibrium of protomeric, diprotomeric, and oligomeric forms of the subunit of $Na^+/K^+$-ATPase within cell membranes during pumping cannot be excluded (31).

The intention of the present work was to get information on the mechanism of the sodium pump and on the interaction of ATP binding sites in catalysis as well as to answer the question as to whether a fluorescent ATP analog may exist which binds with high affinity to the low affinity ATP binding site, the $E_2$ATP site. Therefore, $2’$-O-dansylated ATP derivatives were investigated for their substrate function and their affinities for the ATP binding sites. It turned out that dansylated ATP analogs are not hydrolyzed but interact with the sodium pump with high affinity at a low affinity ATP site. A high affinity of DANS-ATP to the low affinity $E_2$ATP site could be verified by its protective action against the inactivation by CoATP (Fig. 3A). CoATP is an inactivating metal-ATP analog that affects typical reactions of the $E_2$ATP site but does not alter partial activities of the $E_1$ATP site (4). A protective effect against the inactivation of the $E_1$ATP site by CrATP was seen with low affinity of DANS-ATP (Fig. 3B). CrATP is known to arrest the enzyme in an $E_1$, Cr-P state (4). The kinetic constants were evaluated by a modification of the Koshland-Némethy-Filmer model taking into account the specific behavior of the metal-ATP analogs and of the protecting ligands (Fig. 1, Equations 3 and 4). CoATP is known to bind to the $E_1$ATP binding site with high affinity but without inactivation (32). Likewise, we assume no binding of CrATP at the $E_1$ATP binding site without inactivation at this site. However, the strong negative cooperativity in complexes with CrATP ($b = 300, c = 750$) also allows an interpretation without binding of CrATP at the $E_1$ATP binding site. Obviously the kinetics of CrATP and CoATP are special cases of the general two-site model of Koshland-Némethy-Filmer. DANS-N$_3$-ATP inactivated $Na^+/K^+$-ATPase by a biphasic time course (Fig. 4A), and the initial velocity of inac-

![Figure 5](image-url)

**FIG. 5. Effect of DANS-N$_3$-ATP on the partial activities of Na$^+/K^+$-ATPase in modified enzyme.** Panel A, effect of 50 $\mu M$ FITC (●) and 30 $\mu M$ DANS-N$_3$-ATP (■) on the partial activity of the Na$^+$-depend-ment formation of phosphoenzyme in an $E_1$ATP-site blocked (Co(NH$_3$)$_4$PO$_4$-inactivated) Na$^+/K^+$-ATPase. The maximal phosphorylation capacity was 33 pmol/unit. Panel B, effect of 30 $\mu M$ DANS-N$_3$-ATP (■) and 50 $\mu M$ FITC (●) on K$^+$-activated $p$-nitrophenylphosphatase in an $E_1$ATP-site blocked Na$^+/K^+$-ATPase. Inactivation of the $E_2$ATP site was performed by CrAMP-PCP. The maximal activity was 15 pmol/unit. A control with FITC detected no high affinity ATP sites. Panel C, effect of ATP on the inactivation process by DANS-N$_3$-ATP in a CrAMP-PCP-blocked enzyme (●, 150 $\mu M$; 300 $\mu M$ not shown; ●, 600 $\mu M$; ■, no ATP). *Inset*, a replot of the apparent affinities (+S.D.) indicates a binding with a high affinity of $K_I = 10 \pm 3 \mu M$ DANS-N$_3$-ATP. The line was obtained by linear regression analysis.

In contrast a study of the activity changes of K$^+$-activated $p$-nitrophenylphosphatase in an enzyme with a blocked $E_1$ATP site by CrAMP-PCP (12) showed a specific interaction of DANS-N$_3$-ATP with the $E_2$ATP site (Fig. 5B). Again, because of the long incubation time with CrAMP-PCP the poisoned enzyme had less K$^+$-phosphatase activity than the native enzyme. Nevertheless, this activity was inaccessible to 50 $\mu M$ FITC (probe for the $E_2$ATP binding site) (25) but accessible to CoATP (probe for the $E_2$ATP binding site) (13). Provided DANS-N$_3$-ATP interacts with the $E_2$ATP site then ATP should protect K$^+$-activated $p$-nitrophenylphosphatase against the decrease by DANS-N$_3$-ATP. In fact, ATP protected competitively...
tivation showed a sigmoidal concentration dependence (Fig. 4B). One explanation for a biphasic time course is binding of DANS-N$_3$-ATP at two different ATP sites. The modification at one of these sites may not fully inactivate the enzyme. DANS-N$_3$-ATP also decreased the activity of the Na$^+$-dependent protein-phosphokinase and the activity of K$^+$-activated p-nitrophenolphosphatase in the native enzyme (data not shown). Further experiments with E$_1$ATP- and E$_2$ATP-blocked enzyme preparations supported the idea of a higher affinity of DANS-N$_3$-ATP with the E$_2$ATP site. The K$^+$-activated phosphatase was lost in a CrAMP-PCP-enzyme (where the E$_2$ATP site is occupied) with high affinity (Fig. 5B), and the analysis of the competitive protective action of ATP against the DANS-N$_3$-ATP-induced inactivation of K$^+$-activated phosphatase could be determined to occur at a low affinity site for ATP (Fig. 5C, inset). On the contrary, DANS-N$_3$-ATP had no influence on Na$^+$-dependent phosphorylation of the enzyme protein of the E$_1$-ATP site in a Co(NH$_3$)$_4$PO$_4$-inactivated Na$^+/K^+$-ATPase (where the E$_2$ATP site is blocked) (Fig. 5A).

The Koshland-Némethy-Filmer model used here explains our findings by a two-site competitive model with different affinities to the binding sites (Fig. 1). The model presented is similar to a reaction scheme used by the group of Beaugé'to explain their findings with another P-type ATPase, the H$_2$-ATPase. The model presented is our findings by a two-site competitive model with different affinities to the binding sites (Fig. 5C). The model used does not exclude the possibility that the two substrate binding sites reside on a single subunit (30) rather than on neighboring subunits in a (αβ)$_2$ dimer (4, 27, 31). It is an open question so far why ATP analogs like DANS-N$_3$-ATP and DANS-ATP are not substrates for Na$^+/K^+$-ATPase and why the affinities of the enzyme's two substrate binding sites for a specific ATP analog are just the opposite of that of ATP. One may speculate that the specific conformation of the ATP analog may be of importance in this respect and if so, whether some ATP analogs may be hydrolyzed by Na$^+/K^+$-ATPase in a positive cooperative way as well. Preliminary data seem to indicate this (34). It is also noteworthy that Fig. 1 shows only the kinetic states necessary for the description of our data. Thus, each intermediate represents a pool that may be modulated by sodium and potassium ions and allows cooperativity between different conformations in one subunit. The competitive two-site model applies for CrATP, CoATP, and DANS-ATP derivatives. Whether this model explains the inhibitory effects of all ATP analogs and whether it may be useful for additional studies on the enzyme's catalysis of other sub-
strate analogs must be proven by further experiments. In summary, the above findings strongly suggest a high affinity binding of DANS-N\(_3\)-ATP at the low affinity ATP binding site (E\(_A\)-ATP binding site) of Na\(^+\)/K\(^+\)-ATPase which leads to its irreversible inactivation. DANS-N\(_3\)-ATP may also interact with low affinity at the high affinity ATP site (E\(_A\)-ATP site) but in a dissociative way. This peculiar property of DANS-N\(_3\)-ATP makes this fluorescent ATP analog a promising candidate to localize the E\(_A\) site by affinity labeling and peptide sequencing (experiments are in progress). The interaction of DANS-N\(_3\)-ATP and of the other ATP derivatives with Na\(^+\)/K\(^+\)-ATPase can be explained by a Koshland-Nemethy-Filmer model of two interacting ATP sites. This explains positive and negative cooperative interactions of ATP sites with different ATP analogs. It also explains the 30-fold activation of Na\(^+\)/K\(^+\) pump by DANS-N\(_3\)-ATP and of the other ATP derivatives with Na\(^+\)/K\(^+\)-ATPase which leads to its irreversible inactivation. DANS-N\(_3\)-ATP may also interact with Na\(^+\) by affinity labeling and peptide sequencing (experiments are in progress).

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