Tight Binding of Bulky Fluorescent Derivatives of Adenosine to the Low Affinity E2ATP Site Leads to Inhibition of Na+/K+-ATPase

ANALYSIS OF STRUCTURAL REQUIREMENTS OF FLUORESCENT ATP DERIVATIVES WITH A KOSHLAND-NÉMETHY-FILMER MODEL OF TWO INTERACTING ATP SITES

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A Koshland-Némethy-Filmer model of two cooperating ATP sites has previously been shown to explain the kinetics of inhibition of Na+/K+-ATPase (EC 3.6.1.37) by dansylated ATP (Thoenges, D., and Schoner, W. (1997) J. Biol. Chem. 272, 16315–16321). The present work demonstrates that this model adequately describes all types of interactions and kinetics of a number of ATP analogs that differ in their cooperativity of the high and low affinities to ATP binding sites of the enzyme. 2,3′-O(2,4,6-trinitrophenyl)ATP binds in a negative cooperative way to the E1ATP site (Kd = 0.7 μM) and to the E2ATP site (Kd = 210 μM), but 3′(2′)-O-methylanthranilyl-ATP in a positive cooperative way with a lower affinity to the E1ATP binding site (Kd = 200 μM) than to the E2ATP binding site (Kd = 80 μM). 3′(2′)-O(5-Fluoro-2,4-dinitrophenyl)-ATP, however, binds in a noncooperative way, with equal affinities to both ATP binding sites (Kd = 10 μM). In a research for the structural parameters determining ATP site specificity and cooperativity, we became aware that structural flexibility of ribose is necessary for catalysis. Moreover, puckering of the ring atoms in the ribose is essential for the interaction between ATP sites in Na+/K+-ATPase. A number of derivatives of 2′(3′)-O-adenosine with bulky fluorescent substituents bind high affinity to the E2ATP site and inhibit Na+/K+-ATPase activity. Evidently, an increased number of interactions of such a bulky adenosine with the enzyme protein tightens binding to the E2ATP site.

Active Na+/K+-transport through mammalian membrane branes catalyzed by the sodium pump needs the interaction of high and low affinity ATP binding sites during catalysis (1). During pumping, a high affinity ATP site (E1ATP site) is phosphorylated when Na+/K+-ATPase (EC 3.6.1.37) is in its Na+-exporting E1 conformational state. Dephosphorylation, however, turns the enzyme to the K+ importing E2 conformation that binds ATP with low affinity (E2ATP site) (for a review, see Ref. 2). The kinetics of substrate hydrolysis of the enzyme vary with the nature of the nucleoside triphosphate. Although ATP hydrolysis proceeds in a negative cooperative way (3), inhibition of ATP hydrolysis by 2′,3′-O(2,4,6-trinitrophenyl)-ATP (TNP-ATP), a substance that is not hydrolyzed, was reported to be partially competitive and noncompetitive (4). Moreover, 2′(3′)-O(6-N'-N'-dimethylaminonaphthalenesulfonfonyl)-ATP (DANS-ATP) and 8-N2-DANS-ATP, which are not hydrolyzed either, show a positive cooperative effect during interaction with Na+/K+-ATPase (1). MgATP complex analogs can discriminate between E1ATP and E2ATP binding sites (5). Although Cr(H2O)4ATP (Cr-ATP) inactivates the E1ATP binding site, Co(NH3)4ATP (Co-ATP) inactivates the E2ATP site (6–8). The ribosyl-modified TNP-ATP is known as a substance that binds in relation to ATP with increased affinities to both ATP binding sites (4, 9). Furthermore, we showed recently that ribosyl-modified DANS-ATP binds with much higher affinity to the E2ATP site than to the E1ATP site (1). This peculiar phenomenon is not understood very well. A better understanding would be helpful not only to find more protein-reactive ATP derivatives with a preference for the low affinity E2ATP binding site but also to realize whether the method of analysis of the complex kinetics with a Koshland-Némethy-Filmer model of two cooperating ATP sites is generally applicable to all ATP derivatives. Hence, such a model would describe a general property of the enzyme. This would also include that it is justified to extrapolate from the knowledge of microscopic dissociation constants of the E1ATP and E2ATP sites, obtained from the inactivation with MgATP complex analogs (5), to the complex macroscopic kinetics of Na+/K+-ATPase (1).

Therefore, we started a careful kinetic analysis of a number of ATP and nucleoside analogs with modified ribose and polynucleotide moieties. Analysis of all of the substances for their microscopic dissociation constants of the E1ATP and E2ATP sites by previously reported methods (1, 5) and of the kinetics of overall hydrolysis or substrate inhibition by use of a model of two interacting ATP sites revealed that the previously published Koshland-Némethy-Filmer model describes sufficiently well all kinetics. The correlation of kinetic data with structural data led to a postulate of minimal requirements of

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‡ The abbreviations used are: E1ATP site, Cr(H2O)4ATP-sensitive site with high affinity for ATP; E2ATP site, Co(NH3)4ATP-sensitive site with low affinity for ATP; TNP-ATP, 2′,3′-O(2,4,6-trinitrophenyl)ATP-DANS-ATP, 2′(3′)-O(6-N'-N'-dimethylaminonaphthalenesulfonfonyl)-ATP; Cr-ATP, Cr(H2O)4ATP; β,γ bidentate complex of chrom(III) tetraquo-ATP; Co-ATP, Co(NH3)4ATP; β,γ bidentate complex of cobalt(III) tetra
ATP analogs for high affinity interaction with the low affinity E2ATP site. Such properties are a “thickened” adenine ring because of stacking of a ribose-ligated bulky fluorophore at a flexible ribose moiety.

MATERIALS AND METHODS

Chemicals—1-Pyrenesulfonyl chloride, 1-anthracenesulfonyl chloride, and N-methylisatoic anhydride were purchased from Molecular Probes (Eugene, OR). DANS-tryptophan (DANS-TRP) was supplied by Serva (Heidelberg, Germany). Lab-Trol, a protein standard used in clinical chemical analysis, was delivered by Baxter Dade (Dudingen, Switzerland).

Enzyme and Assays—Na\textsubscript{1}/K\textsubscript{1}-ATPase from pig kidneys with a specific activity of 18–25 units/mg was purified by a modification of Jørgensen’s procedure (10) and measured by a coupled optical assay (1). Protein was determined by the method of Lowry et al. (11) using Lab-Trol as a standard. When the inhibitory effect of ATP analogs on Na\textsubscript{1}/K\textsubscript{1}-activated ATP hydrolysis was studied, variable concentrations of all nucleotides were included into the optical assay. For measurement of the hydrolysis of 3'-(2')-O-MANT-ATP, no ATP was added to the optical assay. The reaction was generally started with 0.1 units of Na\textsubscript{1}/K\textsubscript{1}-ATPase.

Synthesis of ATP Analogs—The structures of ATP derivatives used in this study are shown in Fig. 1. 1-N\textsuperscript{6}-Ethenoadenosine 5'-triphosphate (e-ATP) was obtained by a method of Barrio et al. (12). MANT-ATP, MANT-cAMP, and TNP-ATP were prepared according to Hiratsuka and Uchida (13) and Hiratsuka (14). FDNP-ATP, 2'(3')-O-pyrenesulfonoyl-ATP (PYRS-ATP), 2'(3')-O-Anthracenesulfonyl-ATP (ANTS-ATP) and dansylated nucleotides were synthesized by a modified method of Chuan (1, 15, 16). DANS-adenosine was obtained from DANS-AMP by treatment with alkaline phosphatase (17). The purity of the compounds was controlled by thin-layer chromatography (Silica gel 60 F254, 10:6:3 n-butyl alcohol/water/acetic acid) and by UV, fluorescence, and NMR spectroscopy. To determine the concentration of ATP analogs, the amount of ribose and phosphate was analyzed by the orcin test and the method of Fiske and Subarrow, respectively.

Determination of the Microscopic Dissociation Constant of an ATP Analog to a Specific ATP Binding Site of Na\textsubscript{1}/K\textsubscript{1}-ATPase from Its Protective Effect against the Inactivation by Cr-ATP or Co-ATP—Microscopic dissociation constants of the complexes of E\textsubscript{1}ATP and E\textsubscript{2}ATP binding sites with the ATP analogs of interest were estimated from the protective effect of the substances against the inactivation of Na\textsubscript{1}/K\textsubscript{1}-ATPase by Cr-ATP and Co-ATP (1). Cr-ATP has been shown to inactivate by tight binding and by phosphorylation the E\textsubscript{1}ATP site of Na\textsubscript{1}/K\textsubscript{1}-ATPase (6) and Co-ATP by forming a tight complex with the E\textsubscript{2}ATP site (8). 1 unit of Na\textsubscript{1}/K\textsubscript{1}-ATPase was incubated in a total volume of 250 \(\mu\text{L}\) at 37 °C in 60 mM imidazole, HCl, pH 7.25, and increasing concentrations of Cr-ATP (10–100 \(\mu\text{M}\)) or Co-ATP (100–1000 \(\mu\text{M}\)). The inactivation of Na\textsubscript{1}/K\textsubscript{1}-ATPase was recorded in the absence and presence of the respective ATP analog by transferring an aliquot of 20 \(\mu\text{L}\) of the reaction medium in intervals of 15 min to the optical assay. Rate constants of inactivation and dissociation constants of the enzyme-nucleotide complexes were determined by the method of Piskiewicz and Smith (18) and the two-site model (see Equation 2).

Influence of ATP Analogs on the Activity of K\textsuperscript{+}-activated p-Nitrobe-
nymph phosphatase in Native or FITC-treated Na\(^{+}/K\(^{+}\)-ATPase—Na\(^{-}/K\(^{-}\)-ATPase was inactivated by FITC at pH 9 (9) and washed in 50 mM Tris, HCl, pH 7.5. An amount of this enzyme equivalent to 0.1 units of untreated Na\(^{+}/K\(^{+}\)-ATPase or 0.1 units of native enzyme was assayed for K\(^{-}\)-activated phosphatase. Increasing concentrations of p-nitrophenyl phosphate were incubated with 5 mM MgCl\(_2\) and 50 mM KCl in the presence or absence of variable amounts of ATP analogs on microtiter plates at room temperature. The reaction was stopped after 15 min of hydrolysis with 1 N NaOH. Absorbance was measured at 410 nm.

**Kinetic Evaluation of a Two-site Competitive Model of Koshold, Némethy, and Filmer**—A two-site model according to Koshold, Némethy, and Filmer (Fig. 2) was used to analyze the kinetics of ATP analogs (1, 19). The equations for the rate of hydrolysis of substrate S in presence of an inhibitor I (see Equation 1) or the rate of inactivation by an inhibitor I in presence of a protecting ligand S (see Equation 2) were derived according to Segel (20). The latter equation takes into account the specificity of Cr-ATP to the E\(_1\)-ATP binding site (γ = 1, c against infinity) and the specificity of Co-ATP to the E\(_2\)-ATP binding site (γ against infinity). All computations and calculations of binding parameters were performed by use of the program Prism 2.0 of GraphPad Software Inc., San Diego, CA 92121.

The overall reaction in presence of an inhibitor is expressed as

\[ V_{p,\text{max}} = \frac{[S]}{a + K_d} + \frac{[S]^2}{a + 2K_d} + \frac{[I]}{y + K_i} + \frac{[I]^2}{y + 2K_i} + \frac{[I]}{y + K_i} \]

and inactivation by Cr-ATP or Co-ATP in the presence of a ligand is expressed as

\[ V_{p,\text{max}} = \frac{[I]}{y + K_i} + \frac{[I]}{y + K_i} \]

**Determination of Structural Factors in ATP Analogs**—After exchange of the removable protons, the \(^1\)H NMR spectra were recorded in D\(_2\)O in a Bruker AM 400 MHz spectrophotometer. The coupling constants of the protons of ribose allowed an evaluation of the time-averaged structure (21, 22). ATP analogs are termed as flexible if 3 Hz < (\(^J^\text{J}\)= and \(^J^\text{p}\)) < 7 Hz and are termed as fixed if 3 Hz > (\(^J^\text{J}\)= or \(^J^\text{p}\)) > 7 Hz.

Time-resolved fluorescence measurements were performed by the time-correlated single photon-counting method using synchrotron radiation as a source of the excitation light (23). The instrumental function and fluorescence decays were measured sequentially during several 10 of cycles and stored in groups of 2048 channels each (time interval 44.2 ps/channel; total number of counts exceeded 10\(^8\) for each measurement; temperature adjusted to 25 °C by water bath; excitation wavelength λ = 290 nm; emission wavelength λ = 415 nm; excitation and emission bandwidth 9 nm). The total fluorescence decays were collected with the excitation polarizer set to the vertical position and the emission polarizer set at 54.7° (magic angle). A total of 2–3 million counts were collected in each decay, and the maximum entropy method was used for data analysis. The final lifetime distribution was split into as many species as there are peaks separated by two well-defined maxima. The first order averaged lifetime γ was then calculated as Σ γ_\(\text{r}\). Errors on averaged lifetimes are based on estimates of the repeatability of the measurements.

**RESULTS**

**Analysis of the Interaction of TNP-ATP with High and Low Affinity ATP Binding Sites of Na\(^{+}/K\(^{+}\)-ATPase**—To learn whether the knowledge of the microscopic dissociation constant of the enzyme complex with an ATP derivative at a specific ATP site may facilitate the kinetic analysis of the overall reaction according to the Koshold-Némethy-Filmer model, the interaction of TNP-ATP with Na\(^{+}/K\(^{+}\)-ATPase was evaluated. Affinities of TNP-ATP for the two substrate binding sites (Fig. 2) were determined from their protective effect against the inactivation by Cr-ATP and Co-ATP (Fig. 3). The \(K_d\) = 0.7 ± 0.3 μM and a = 300 ± 100 of TNP-ATP obtained by the fitting process (Equation 2) to the data analyzed with both inactivating MgATP analogs is in good agreement with direct measurements in the native enzyme (4). Furthermore, the analysis of inactivation of TNP-ATP on the hydrolysis of p-nitrophenyl phosphate with \(K_i\) = 160 ± 20 μM in a FITC-treated enzyme but with \(K_i\) = 7 ± 2 μM in native enzyme is indicative for negative cooperativity (Fig. 4). ATP hydrolysis by Na\(^{-}/K\(^{-}\)-ATPase also shows negative cooperativity (3). The complex kinetics of ATP hydrolysis in the presence of TNP-ATP could not be described quantitatively so far (4). When the two-site model (Fig. 2, Equation 1) was applied to fit curves to experimental points, an excellent fit was obtained (Fig. 5). Using the \(K_i\) values of TNP-ATP as determined above for both ATP sites as microscopic dissociation constants, the experimental finding of downward-bending lines in the double reciprocal plot was quantitatively described by the parameters \(K_d(\text{ATP})\) = 0.3 ± 0.1 μM, \(K_i(\text{TNP-ATP})\) = 0.1 ± 0.05 μM, a = 400 ± 100, b = 70 ±
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The affinities of TNP-ATP for the ATP sites were determined from the effect against the inactivation by Cr-ATP (top) and Co-ATP (bottom). It is noteworthy that TNP-ATP protects against the inactivation by Cr-ATP and that it accelerates the inactivation by Co-ATP at low concentrations of TNP-ATP. Both cases, however, are described by the same microscopic dissociation constants. The following TNP-ATP concentrations were included into the inactivation assay for Cr-ATP: ○, 0.5 μM; ▲, 1 μM; □, 3 μM; ●, 5 μM; ■, no TNP-ATP; and for Co-ATP: ○, 50 μM; ▲, 100 μM; □, 200 μM; ■, no TNP-ATP. The ordinates were normalized to the maximal inactivation rate constant.

One typical experiment is shown. The lines and parameters are the result of fitting of the data according to the Koshland-Némethy-Filmer model (Equation 2):

\[ K_{d}(Cr-ATP) = 0.6 \pm 0.2 \text{ μM}, \quad c = 500 \pm 200, \]

\[ K_{d}(Cr-ATP) = 20 \pm 3 \text{ μM}, \quad K_{d}(TNP-ATP) = 0.7 \pm 0.3 \text{ μM}, \quad a = 300 \pm 100. \]

30, c = 2000 ± 500, and z = 8 ± 4 (Fig. 5). Hence, the knowledge of microscopic dissociation constants for the two ATP binding sites facilitates the fitting of kinetic data of the overall reaction, considerably. Because this procedure was already applied successfully in a previous study to describe the interaction of another ATP derivative with Na\(^+/\)K\(^+\)-ATPase (1), this procedure seems to be valid generally. Therefore, it was applied in the subsequent study as well.

**Analysis of the Interaction of MANT-ATP and FDNP-ATP with High and Low Affinity ATP Binding Sites of Na\(^+/\)K\(^+\)-ATPase**—In contrast to TNP-ATP and DANS-ATP (1, 4) other ribose-modified ATP analogs like MANT-ATP and FDNP-ATP are substrates of Na\(^+/\)K\(^+\)-ATPase (14) (Table I). A careful analysis of the E1ATP- and E2ATP sites, as described above, showed for FDNP-ATP identical affinities for both substrate binding sites (K\(_d\) = 10 μM) and, hence, a Michaelis-Menten type of hydrolysis (data not shown). MANT-ATP, however, exhibited a positive cooperativity with a K\(_d\) = 200 ± 50 μM for the E1ATP site and a K\(_d\) = 80 ± 40 μM for the E2ATP site. This result could be corroborated by an analysis of the effect of MANT-ATP on K\(^+\)-activated p-nitrophenylphosphatase in a E2ATP site-blocked enzyme. It gave for MANT-ATP the K\(_d\) = 100 ± 20 μM in a FITC-treated enzyme and K\(_d\) = 300 ± 50 μM in an untreated enzyme (data not shown). Apparently, modification of the E1ATP binding site by the adenine-imitating FITC (to which MANT-ATP binds with low affinity) enhances in a positive cooperative way binding of MANT-ATP to the E2ATP site.

When the overall kinetics of substrate hydrolysis were analyzed with the Koshland-Némethy-Filmer model (Equation 1), the following parameters were obtained by the fitting process: for MANT-ATP, K\(_d\) = 200 ± 50 μM, a = 0.4 ± 0.2, z = 0.3 ± 0.2; for FDNP-ATP, K\(_d\) = 5 ± 2 μM, a = 1 ± 0.3, c = 1 ± 0.5; and for ATP, K\(_d\) = 1 ± 0.2 μM, a = 100 ± 20, z = 15 ± 5 (Fig. 6).

Hence, the individual ATP derivatives do not only differ in their individual affinities for the two ATP binding sites (Table I) but also in the turnover rate (z value). The maximal velocity of Na\(^+/\)K\(^+\)-supported hydrolysis of ATP (V\(_{\text{max}}\) = 30 units/ml) is about 15 times faster than that of FDNP-ATP and about 50 times faster than that of MANT-ATP.

The Hill coefficient is a measure of cooperativity. It is calculated as the first derivative of Equation 1 as log(1 + v)/log(1 + v) against log(S). For the Koshland-Némethy-Filmer model of two interacting substrate sites, this derivative is shown in Equation 3. When the above-evaluated data were used to calculate the change of cooperativity as a function of substrate concentration, Fig. 7 resulted. It is well known from the work of Cornish-Bowden and Koshland (25) that the cooperativity changes with the substrate concentration. Evidently negative cooperativity of ATP was most pronounced at very low ATP concentrations, i.e., at ATP concentrations that are commonly

**FIG. 4. Analysis of K\(^+\)-activated hydrolysis of p-nitrophenylphosphatase in the presence of TNP-ATP.** The effect of various concentrations of TNP-ATP on the activity of K\(^+\)-activated p-nitrophenylphosphatase was measured in native (top) and FITC-treated Na\(^+/\)K\(^+\)-ATPase (bottom). Treatment of Na\(^+/\)K\(^+\)-ATPase by FITC is known to block the Cr-ATP-sensitive E1ATP binding site but not the Co-ATP sensitive E2ATP binding site (7). The following concentrations of TNP-ATP were included into the phosphatase assay for native enzyme: ○, 10 μM; ▲, 50 μM; □, 100 μM; ■, 200 μM; ■, no TNP-ATP. One typical experiment is shown. Inset, replot of the apparent affinities of potassium phosphatase for p-nitrophenyl phosphate against the TNP-ATP concentration. The K\(_d\) (TNP-ATP) = 7 ± 2 μM in native enzyme and the K\(_d\) (TNP-ATP) = 160 ± 20 μM in FITC-treated enzyme were extrapolated from the intercept of the straight line with the abscissa. U, units.
used for the demonstration of Na⁺/K⁺-dependent phosphorylation (26). Higher concentrations of ATP are known to affect the hydrolysis of the phospho intermediate (27, 28).

Analysis of the E₁ATP Site Specificity of Ribose-modified Fluorescent ATP Derivatives—It is unclear why the ribosyl-modified 2′(3′)-O-DANS-ATP and 3′(2′)-O-MANT-ATP bind with higher affinity to the E₁ATP site than to the E₂ATP site (1) and why 2′,3′-O-TNP-ATP preferentially interacts with the E₁ATP site (Table I). Moreover, it is unclear why TNP-ATP and possible answer for the E₁ATP site specificity is that a bulky fluorescent substituent at the ribose may achieve a better affinity for this site. In the case of oxidative phosphorylation, the particular behavior of DANS-ADP was explained by a hydrophobic (charge-transfer, stacking) complex between the ade-
nine and dansyl moiety (29). To test this hypothesis, a number of dansylated nucleotides were synthesized and studied on the overall reaction as well as for their protective effect against the inactivation of the E1ATP site by Cr-ATP and the E2ATP site by Co-ATP (Table I). Consistent with the above hypothesis, dansylated purine triphosphates like DANS-ATP and DANS-GTP showed a preference for the E2ATP site, but the pyrimidine derivative DANS-CTP did not. Because all dansylated derivatives of adenosine (DANS-ATP, DANS-AMP, and DANS-adenosine) except 2'-O-DANS-3',5'-cyclic AMP (DANS-cAMP) showed a preferential binding to the E2ATP site, it was in fact possible that interaction of a bulky residue with the purine part is of importance for the E2ATP site specificity. To get additional information on the validity of this assumption, PYRS-ATP and ANTS-ATP were synthesized and investigated. In fact, ANTS-ATP and PYRS-ATP behaved like DANS-ATP (Table I). Because neither DANS-cAMP and MANT-cAMP nor dansylated tryptophan showed this specificity for the E2ATP site, it is evident that the interaction between the adenosine and the fluorophore per se is not responsible for the E2ATP site specificity but that, additionally, a characteristic puckering of the ribose moiety in the fluorescent nucleoside is needed to achieve a binding at the E2ATP site. In this context 3',5'-cyclo-AMP is known to exhibit a stable C2endo conformation of its ribose because of the intramolecular 3',5' phosphodiester bond (21). TNP-ATP, furthermore, may have a stable conformation of its ribose because of the formation of a Meisenheimer complex of the trinitrophenyl residue with the 2' and 3' hydroxyl groups (Fig. 1). In other derivatives, however, the ribose shows several conformations because its puckering is not restricted by the attached fluorophore (21).

1H NMR Studies and Dynamic Fluorescent Measurements of ATP Analogos—The above findings seem to indicate that the nature and conformational flexibility or stability of a given ATP derivative is of importance for its ATP site specificity and also for the question of whether a given ATP analog is a substrate or an inhibitor. The specificity for the binding sites in turn determines the degree of interaction between ATP sites. To get more reliable and independent data on the structure and conformational dynamics of the ribose moiety of some important ATP derivatives, we analyzed them by 1H NMR and dynamic fluorescence spectroscopy. 1H NMR measurements in D2O revealed changes of the coupling constants of substituted ribose atoms C2' because of attached fluorescent residues (Table II). Analysis of the data according to the concept of pseudorotation (21, 30) showed that ribose in ATP, MANT-ATP, FDNP-ATP, and DANS-ATP is flexible and oscillates between the two major conformations, C2endox and C2endox (21) (Fig. 1). TNP-ATP and DANS-cAMP, however, had a fixed ribose in a specific conformation because of the 2',3'-O-trinitrophenyl group of the Meisenheimer complex and the bridge of the 3',5'-diphos-
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The ATP analogs for the E1ATP and E2ATP sites (obtained from was carried out (Tables 1–3). The comparison of the affinities of a number of ribosyl-modified fluorescent adenosine derivatives drawn in the envelope conformation obtained by 1H NMR and fluorescence spectroscopy. The ribose was drawn in the envelope conformation \(\delta_\text{endo}\) for TNP-ATP (top), in the \(\chi_\text{endo}\) conformation for DANS-cAMP (middle), and in the \(\chi_\text{endo}\) conformation for DANS-ATP (bottom). The dansyl residue was orientated in proximity to the adenine base. Energy minimization (see "Materials and Methods") was performed to the structures to get information about whether the conformational parameters of ATP analogs may correlate to the corresponding binding affinities at the ATP sites of Na\(^+\)/K\(^-\)-ATPase. Obviously, TNP-ATP exhibits negative cooperativity (high affinity to the E1ATP site) and a flat adenine moiety, whereas DANS-ATP exhibits positive cooperativity (high affinity to the E1ATP site) and a "bulky adenine." Probably, these conformations represent the corresponding structures for high affinity binding to the individual ATP sites.

Scopic dissociation constants for its analysis allowed it to describe quantitatively the peculiar behavior of ATP and of its analogs to induce all types of cooperativity in hydrolysis and inhibition of Na\(^+\)/K\(^-\)-ATPase. Because the structural reasons for the variation in cooperativity and in the specificity of substrate sites were unclear but of considerable interest for the understanding of the mechanism of the sodium pump, a more detailed analysis on the interaction of Na\(^+\)/K\(^-\)-ATPase with a number of ribosyl-modified fluorescent adenosine derivatives was carried out (Tables 1–3). The comparison of the affinities of the ATP analogs for the E1ATP and E2ATP sites (obtained from their protective effects against the inactivation of Na\(^+\)/K\(^-\)-ATPase by either Cr-ATP or Co-ATP) revealed that substitution at the hydroxyl groups of the ribose in ATP by bulky fluorescent substituents like dansyl, pyrenesulfonyl, or anthracenesulfonyl residues led to a preferential binding to the E2ATP site (Table I). An interaction with the E2ATP site occurred also when the \(\gamma\)-phosphate or C9\(-\)-phosphate was missing, because DANS-AMP and DANS-adenosine bound with high affinity to this site as well. But a bulky residue alone does not seem to be sufficient to achieve high affinity interaction with the E2ATP site, because DANS-GTP and DANS-CTP interfered only weakly with this site and DANS-TRP interacted with the E1ATP site (Table I). Apparently, there is need of the adenine moiety. The fluorescent 2\'-O-DANS-3',5'-cyclic-AMP also did not interfere at all with any ATP site (Table I), although this compound showed the most pronounced effect in energy transfer (and hence stacking) between the adenine and the dansyl residue (Table III). Obviously, there are additional structural parameters determining the E2ATP site specificity of DANS-ATP, DANS-AMP, DANS-adenosine, ANTS-ATP, and PYRS-ATP (Table I). The ribose conformation in those compounds is certainly an important major additional determinant for the ATP site specificity and cooperativity in Na\(^+\)/K\(^-\)-ATPase. The concept of pseudorotation (30) allows description of the influence of substituents on the structure of the ATP molecule. The ribose in unsubstituted ATP exists in at least two preferred twisted conformations (\(C_2\)-endo and \(C_3\)-endo) (21). There is a low energy barrier between these conformations, and the atoms seem to migrate (pseudorotate) around the ribose. The phosphate chain and the adenine base are substituents of the ribose and can be interpreted as two masses flickering on a puckered basis. A fluorescent substituent of the ribose acts as a third mass and stabilizes a ribose conformation by affecting the equilibrium between the various puckered ribose conformations. It is able to stabilize a conformation and to prevent the pseudorotation between twisted conformations (21). Information on the flexibility of the ribose and on the extent of pseudorotation was obtained by 1H NMR spectroscopy. The analysis of the spectra and the coupling constants revealed that DANS-cAMP has a fixed ribose (Table II). But a fixed ribose does not seem to decide whether an ATP derivative interferes with a specific ATP site or not. TNP-ATP, which has a fixed ribose conformation as well (Table II), shows a much higher affinity for the E1ATP than for the E2ATP site (Table I). Therefore, ribose puckering may differ in both molecules. TNP-ATP, with its inflexible ribose moiety, is not hydrolyzed by Na\(^+\)/K\(^-\)-ATPase (4). Hence conformational flexibility of the ribose moiety seems of importance for the catalytic process. Other ATP derivatives showing puckering of their ribose despite of its substitution (Table II) are substrates of the enzyme but with a much reduced turnover rate, namely MANT-ATP and FDNP-ATP. They bind with high affinity to the E2ATP binding site (Table I). The theory of transition states in enzyme catalysis explains increasing affinities of a ligand to its sites by a greater structural identity to the enzyme-substrate complex. This better binding, however, is payed for by a decrease in the following rate-determining steps (31, 32). It is probable, therefore, that ATP analogs with a higher affinity to the E2ATP binding site than the natural substrate ATP are much more slowly hydrolyzed by Na\(^+\)/K\(^-\)-ATPase. Hence, DANS-ATP, showing a flexible ribose but with a very high affinity for the E2ATP site inducing the strongest positive cooperativity, is, unlike MANT-ATP and FDNP-ATP, not a substrate of Na\(^+\)/K\(^-\)-ATPase. The same allosteric effect may be employed to explain DANS-ATP protection against Cr-ATP, because binding of DANS-ATP was shown to be fully reversible (1) and binding of Cr-ATP to the E1ATP site was not detectable at the low concentrations used in the experiment (data not shown). ATP, FDNP-ATP, and MANT-ATP apparently undergo changes in their ribose conformations during catalysis. They seem to be able to switch their ribose pucker from a conformation that binds to the E1ATP site

![Figure 8: Structures of TNP-ATP, DANS-cAMP, and DANS-ATP.](http://www.jbc.org/)
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(which in turn induce the E\(_2\) ATP binding site) to a conformation that dissociates from the E\(_2\) ATP binding site. Such a sequential inductive process is independent of the steric arrangement of the ATP sites on the enzyme protein (33) and is consistent with the two-site model of Koshland, Némethy, and Filmer (1). This means also that binding of ATP analogs to the E\(_2\) ATP site is only possible after binding to the E\(_1\) ATP binding site regardless of the question of the localization of these ATP sites on one or more protein subunits (34). Evidently, this is seen in the case of DANS-cAMP, DANS-TRP, DANS-GTP, and DANS-CTP that cannot bind to the E\(_1\) ATP site and, in turn, not to the E\(_2\) ATP site because of the change in the phosphate region, the lack of ribose pucker, or the missing adenosine moiety.

In summary the ATP binding sites in Na\(^+/K^+\)-ATPase show all types of cooperativity in their interaction with ATP analogs. A two-site model according to Koshland, Némethy, and Filmer is able to explain the kinetic behavior of all substrates and inhibitors of Na\(^+/K^+\)-ATPase (Figs. 3–7). There is a high degree of probability that within the adenosine moiety, the ribose conformation in ATP analogs bears the information responsible for its preferential affinity and cooperativity between the ATP sites of Na\(^+/K^+\)-ATPase. Obviously, free pseudorotation is necessary for the hydrolysis of ATP analogs, whereas restriction in a specific ribose conformation leads to preferential binding to one of the two ATP sites in Na\(^+/K^+\)-ATPase. A bulky fluorescent mass at the 2’(3’) position of the ribose that interacts with the adenin moiety apparently supports high affinity binding at the E\(_2\) ATP site of Na\(^+/K^+\)-ATPase (Fig. 8). A simplified explanation for this finding is that more protein substrate contacts exist within the E\(_2\) ATP site with a bulky substrate, which hence lead to a tighter binding of this more open ATP site in its E\(_2\) conformation to a more voluminous ATP molecule. The cavity of the ATP site in its E\(_1\) conformation, on the one hand, is too small to interact with such bulky ATP analogs (35). The flexibility of ribose, on the other hand, allows structural rearrangements of the ATP molecule and subsequently binding to the E\(_1\) ATP site with low affinity. This is a prerequisite for high affinity substrate binding at the E\(_2\) ATP site (according to the Koshland-Némethy-Filmer model of induced fit) and for the induction of positive cooperativity. With this knowledge of structural substrate requirements of each of the ATP sites of Na\(^+/K^+\)-ATPase it should now be possible to construct protein-reactive ATP derivatives that allow detection of the amino acids forming the low affinity E\(_2\) ATP site.

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Tight Binding of Bulky Fluorescent Derivatives of Adenosine to the Low Affinity E$_2$ ATP Site Leads to Inhibition of Na$^+$/K$^+$-ATPase: ANALYSIS OF STRUCTURAL REQUIREMENTS OF FLUORESCENT ATP DERIVATIVES WITH A KOSHLAND-NÉMETHY-FILMER MODEL OF TWO INTERACTING ATP SITES

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