Characterization of 2',3'-O-(2,4,6-Trinitrocyclohexadienylidine)adenosine 5'-Triphosphate as a Fluorescent Probe of the ATP Site of Sodium and Potassium Transport Adenosine Triphosphatase

DETERMINATION OF NUCLEOTIDE BINDING STOICHIOMETRY AND ION-INDUCED CHANGES IN AFFINITY FOR ATP*

(Received for publication, March 28, 1980, and in revised form, November 13, 1980)

Edward G. Moczydlowski§ and P. A. George Fortes§
From the Department of Biology, C-016, University of California, San Diego, La Jolla, California 92039

The fluorescent ATP derivative 2',3'-O-(2,4,6-trinitrocyclohexadienylidine) adenosine 5'-triphosphate (TNP-ATP) binds specifically with enhanced fluorescence to the ATP site of purified eel electroplax sodium-potassium adenosine triphosphatase, (Na,K)-ATPase. A single homogeneous high affinity TNP-ATP binding site with a $K_d$ of 0.04 to 0.09 $\mu$m at 3°C and 0.2 to 0.7 $\mu$m at 21°C to 25°C was observed in the absence of ligands when binding was measured by fluorescence titration or with [$^3$H]TNP-ATP. ATP and other nucleotides competed with TNP-ATP for binding with $K_d$ values similar to those previously determined for binding to the ATP site. Binding stoichiometries determined from Scatchard plot intercepts gave one TNP-ATP site/175,000 g of protein (range: 1.64 x 10$^4$ to 1.92 x 10$^4$) when (Na,K)-ATPase protein was determined by quantitative amino acid analysis. The ratio of [H]ouabain sites to TNP-ATP sites was 0.91. These results are inconsistent with "half-of-sites" binding and suggest that there is one ATP and one ouabain site/β protomer. (Na,K)-ATPase maintained a high affinity for TNP-ATP regardless of the ligands present. K+ increased the $K_d$ for TNP-ATP about 5-fold and Na+ reversed the effect of K+. The effects of Na+, K+, and Mg$^{2+}$ on ATP binding at 3°C were studied fluorimetrically by displacement of TNP-ATP by ATP. The results are consistent with competition between ATP and TNP-ATP for binding at a single site regardless of the metallic ions present. The derived $K_d$ values for ATP were: no ligands, 1 $\mu$m; 20 mM NaCl, 3-4 $\mu$m; 20 mM KCl, 15-19 $\mu$m; 20 mM KCl + 4 mM MgCl$_2$, 70-120 $\mu$m. These results suggest that a single ATP site exhibits a high or low affinity for ATP depending on the ligands present, so that high and low affinity ATP sites observed kinetically are interconvertible and do not co-exist independently. We propose that during turnover the affinity for ATP changes more than 100-fold owing to the conformational changes associated with ion binding, translocation, and release.

ATP plays a dual functional role in the active transport of Na$^+$ and K$^+$ by (Na,K)-ATPase: 1) In the presence of Na$^+$, ATP binds with high affinity ($K_d = 0.1-1 \mu$m, Refs. 1-3) to a catalytic site involved in enzyme phosphorylation and outward Na$^+$ transport (4-6); 2) ATP activates enzyme turnover with low affinity ($K_d = 0.1-0.4 \mu$m) at a regulatory site in the presence of Na$^+$ plus K$^+$. In its low affinity role, ATP is thought to promote the intracellular release of K$^+$ initially bound externally (4, 6-13). It has also been suggested that the functional enzymatic unit of (Na,K)-ATPase is a diprotomer of catalytic subunits only one of which can be phosphorylated or bind ATP or ouabain at any given time (3, 14-19). Proposed anticooperative interactions between subunits or alternating site models (3, 17, 20-24) imply co-existence of high and low affinity ATP sites, one on each protomer of one diprotomer. However, the binding of ATP to the low affinity site has not been measured directly for technical reasons. In order to test the co-existence of high and low affinity sites, we have studied the interaction of (Na,K)-ATPase with TNP-ATP (Fig. 1), a fluorescent ATP derivative first used as a spectroscopic probe of the ATP site in myosin (25). Here we characterize TNP-ATP as a specific fluorescent ligand of the ATP site in purified eel electroplax (Na,K)-ATPase. We report nucleotide binding stoichiometries and use TNP-ATP fluorescence to measure the affinity of (Na,K)-ATPase for ATP under various ligand conditions. In the accompanying paper, (27) the effect of TNP-ATP on (Na,K)-ATPase activity and a mechanism for (Na,K)-ATPase consistent with the present results are presented. Preliminary communications of parts of this work have been previously presented (28-30).

* This work was supported by National Institutes of Health Grants HL-20262 and RR-08135 and Grant-in-Aid 74-1072 from the American Heart Association. Preliminary communications of parts of this work were presented at the 30th annual meeting of the Society of General Physiologists, Sept. 22 to 26, 1977 and the 2nd International Conference on the (Na,K)-ATPase, Sandbjerg, Denmark, Sept. 17 to 21, 1978. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by United States Public Health Service Predoctoral Training Grant GM 07313-ST2. This work is part of a thesis submitted to the University of California, San Diego in partial fulfillment of the requirements for the Ph.D. degree. Present address, Pharmacology Department, Harvard Medical School, Boston, MA 02115.

To whom correspondence should be addressed.

1 The abbreviations used are: (Na,K)-ATPase, sodium-potassium transport adenosine triphosphatase (EC 3.6.1.3); AMP-PCP, adenylyl-5'-yl (β,γ-methylene)-diphosphonate; AMP-PNP, adenylyl-5'-yl-5'-imidodiphosphate; TNP-ATP, 2',3'-O-(2,4,6-trinitrocyclohexadienylidine)adenosine 5'-triphosphate; CDTA, trans-1,2-diaminocyclohexane-N,N',N'-tetraacetate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate, $K_d$, dissociation constant; $K_{app}$, apparent dissociation constant; $\lambda_{ex}$, $\lambda_{em}$, excitation or emission wavelength.
**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation**—Eel electroplax (Na,K)-ATPase was purified either by zonal centrifugation (16, 32), as described previously (33) except that no asolectin was added to the Lubrol extract, or by aminoethyl cellulose chromatography (34). After ammonium sulfate precipitation and centrifugation, the pellets of purified enzyme were resuspended in 1 mM Tris-HCl and 0.1 mM CDTA, pH 7.0, with a glass homogenizer, and aliquots were frozen in liquid N₂ and stored at −80°C.

No significant differences in specific activity, gel electrophoresis patterns, or TNP-ATP binding capacities were seen between the enzymes purified by zonal centrifugation or chromatography. Approximately 90–95% of the protein consisted of the α and β polypeptides with apparent Mᵦ of about 95,000 and 50,000 based on Coomassie blue staining of SDS gels. The specific activity, assayed as described previously (35), ranged from 35–49 μmol of ATP hydrolyzed min⁻¹ mg⁻¹ based on protein determined by amino acid analysis. The binding studies reported here were carried out with the most active preparations.

**Protein Determinations**—(Na,K)-ATPase protein was determined by both quantitative amino acid analysis and by the method of Lowry et al. (36), as described by Markwell et al. (37), using bovine serum albumin as a standard. The concentration of albumin used in the Lowry assay was also determined by amino acid analysis using the number of residues of each amino acid based on the published sequence (38). Quantitative amino acid analysis of albumin and (Na,K)-ATPase was performed after 24-h hydrolysis of the protein samples at 110°C in 6 N HCl. Forty μmol of norleucine was added to each sample before hydrolysis as an internal standard for the fraction of the original sample applied to the analyzer. The hydrolyzed samples were analyzed on a Beckman 118C single column amino acid analyzer.

The concentration of each amino acid except Pro, Trp, Met, and Cys was determined. These amino acids constitute 9.9% and 10.7% of the other amino acids gave the same average values as those of Perrone et al. (16) with a maximum difference of 1 mol/100 mol of amino acid in the case of Glu, Leu, and Phe. Therefore, the total weight of the protein samples was estimated as the sum of all the measured amino acids plus 11.5% to account for the weight contribution of Pro, Trp, Met, and Cys.

**Synthesis of [3H]TNP-ATP**—The synthesis followed the method of Hiratsuka and Uchida (25) for unlabeled TNP-ATP. Radioactive ATP (32.3 mmol, 27.9 Ci/mmol of [3,5,7-3H]ATP) was diluted with unlabeled Na₂ATP (89 μmol). The pH was maintained at 9.5 with LiOH with a Radiometer TTT60 titrator. Thirty-six pmol of 2,4,6-trinitrobenzene sulfonic acid was added slowly from a 1 M solution over a period of 2 h. The reaction was allowed to progress for 68 h at 21°C. The reaction mixture was chromatographed on a column of LH-20 Sephadex eluted with H₂O. The fractions were assayed for radioactivity and a separation profile similar to that described (25) was obtained. Only the leading edge of the TNP-ATP peak was pooled since the trailing edge was not well separated from TNP-ADP. The product, analyzed by paper chromatography in 1-butanol/acetic acid/H₂O (40:10:25, ascending) and butyric acid/ammonium hydroxide/0.1 M EDTA/H₂O (66:1:32, descending), was chromatographically pure as indicated by a single yellow fluorescent spot and the absence of [3H]ATP or [3H]ADP by assay for radioactivity. The yield of [3H]TNP-ATP was approximately 90% on the basis of total TNP-ATP in the reaction. The final specific activity of the purified material was 73.5 mCi/mmol. Twenty-six per cent of the tritium label was lost during the reaction, apparently due to the high pH. Using the extinction coefficients of 2.64 × 10⁻² M⁻¹ cm⁻¹ at 408 nm and 1.85 × 10⁻² M⁻¹ cm⁻¹ at 470 nm (26), we estimated that the product contained 2.09 ± 0.02 mol of acid-labile (10 min at 100°C in 1 N HCl) inorganic phosphate/mol of trinitrocyclohexadienide anion. Inorganic phosphate was analyzed by the method of Sanui (40). No loss of tritium or absorbance at 408 nm or 470 nm in the product was found over a 5-month period when it was stored at 0°C in water (H₂O).

**Direct Binding Assays**—[3H]TNP-ATP and [3H]ouabain binding were measured in duplicate samples at each concentration either by the gel equilibrium method of Hirose and Kano (41) or by the forced dialysis method of Cantley and Hammes (42), with the following modifications.

In the gel equilibrium method, enzyme, radioactive ligand, and other additions were made in a final volume of 0.8 ml to tubes containing 50 mg of dry Sephadex G-50 swollen with 0.75 ml of buffer. The tubes were incubated with stirring for a minimum of 30 min at room temperature (23 ± 2°C) in the case of TNP-ATP and 90 min in the case of ouabain, centrifuged in a clinical centrifuge, and 50–100 μl of the supernatant was assayed for radioactivity by liquid scintillation counting.

In the forced dialysis method, the enzyme, plus radioactive ligand was incubated in a final volume of 0.4 ml. Bound ligand was calculated as the difference between the total ligand added and the free ligand measured by radioactivity in the first 20 μl of filtrate. Nucleopore polycarbonate filters with a 0.88 μm pore size were ideal for binding assays since this filter passed less than 1% of the total tritophan fluorescence of purified eel (Na,K)-ATPase and >96% of the radioactivity of [3H]TNP-ATP or [3H]ouabain was recovered after filtration in the absence of enzyme or with a 200-fold excess of unlabeled TNP-ATP or ouabain, indicating that nonspecific binding was negligible for these ligands. The percentage of total ligand that was bound ranged from 5% to 90% in the titrations.

**Absorbance Measurements**—Spectra in the absence of (Na,K)-ATPase were recorded with a modified Cary 14 spectrophotometer (43) in which the sample (0.5 ml) was placed directly on top of the photomultiplier tube and illuminated from the top. This arrangement allowed detection of all the transmitted light regardless of the turbidity of the sample.

**Fluorescence Measurements**—The instrumental set-up for fluorescence measurements and corrected fluorescence spectra was as previously described (35). All TNP-ATP fluorescence titrations were samples of ATPase containing 50 μg of dry Sephadex G-50 swollen with 0.75 ml of buffer. The product was measured in dimethylformamide and dimethylsulfoxide in 0.1 N NaOH as a standard of quantum yield 0.92 (44).

Fluorescence polarization spectra were corrected for instrumental polarization which were measured as described previously (33) either in the Perkin Elmer MPR-4 spectrofluorometer or in a photon-counting instrument in Dr. G. Weber's laboratory at the University of Illinois. Excitation was with a 2- to 5-nm band pass in some titrations, a Corning 5-60 filter in the excitation path and a Corning 3-71 cut-off filter in the emission path were used to minimize stray and scattered light. The absolute quantum yield of TNP-ATP in dimethylformamide was measured as described previously (33) using fluorescein in 0.1 N NaOH as a standard of quantum yield 0.92 (44).

The fluorescence polarization spectra were corrected for instrumental polarization which were measured as described previously (33) either in the Perkin Elmer MPR-4 spectrofluorometer or in a photon-counting instrument in Dr. G. Weber's laboratory at the University of Illinois. Excitation was with a 2- to 5-nm band pass in some titrations, a Corning 5-60 filter in the excitation path and a Corning 3-71 cut-off filter in the emission path were used to minimize stray and scattered light. The absolute quantum yield of TNP-ATP in dimethylformamide was measured as described previously (33) using fluorescein in 0.1 N NaOH as a standard of quantum yield 0.92 (44).

Fluorescence polarization spectra were corrected for instrumental polarization which were measured as described previously (33) either in the Perkin Elmer MPR-4 spectrofluorometer or in a photon-counting instrument in Dr. G. Weber's laboratory at the University of Illinois. Excitation was with a 2- to 5-nm band pass in some titrations, a Corning 5-60 filter in the excitation path and a Corning 3-71 cut-off filter in the emission path were used to minimize stray and scattered light. The absolute quantum yield of TNP-ATP in dimethylformamide was measured as described previously (33) using fluorescein in 0.1 N NaOH as a standard of quantum yield 0.92 (44).

Fluorescence polarization spectra were corrected for instrumental polarization which were measured as described previously (33) either in the Perkin Elmer MPR-4 spectrofluorometer or in a photon-counting instrument in Dr. G. Weber's laboratory at the University of Illinois. Excitation was with a 2- to 5-nm band pass in some titrations, a Corning 5-60 filter in the excitation path and a Corning 3-71 cut-off filter in the emission path were used to minimize stray and scattered light. The absolute quantum yield of TNP-ATP in dimethylformamide was measured as described previously (33) using fluorescein in 0.1 N NaOH as a standard of quantum yield 0.92 (44).

Fluorescence polarization spectra were corrected for instrumental polarization which were measured as described previously (33) either in the Perkin Elmer MPR-4 spectrofluorometer or in a photon-counting instrument in Dr. G. Weber's laboratory at the University of Illinois. Excitation was with a 2- to 5-nm band pass in some titrations, a Corning 5-60 filter in the excitation path and a Corning 3-71 cut-off filter in the emission path were used to minimize stray and scattered light. The absolute quantum yield of TNP-ATP in dimethylformamide was measured as described previously (33) using fluorescein in 0.1 N NaOH as a standard of quantum yield 0.92 (44).

Fluorescence polarization spectra were corrected for instrumental polarization which were measured as described previously (33) either in the Perkin Elmer MPR-4 spectrofluorometer or in a photon-counting instrument in Dr. G. Weber's laboratory at the University of Illinois. Excitation was with a 2- to 5-nm band pass in some titrations, a Corning 5-60 filter in the excitation path and a Corning 3-71 cut-off filter in the emission path were used to minimize stray and scattered light. The absolute quantum yield of TNP-ATP in dimethylformamide was measured as described previously (33) using fluorescein in 0.1 N NaOH as a standard of quantum yield 0.92 (44).
The presence and absence of enzyme. In this experiment, approaches that in buffer alone. The fluorescence enhancement suggesting it reflects TNP-ATP binding to a nucleotide site was prevented or reversed by addition of 2-4 mM ATP, as discussed in the next section.

Fluorescence enhancements were plotted as a function of the total TNP-ATP concentration. Consecutive TNP-ATP additions were made to the same sample with increasing amounts of enzyme and by measuring fluorescence and binding in the sample using [3H]TNP-ATP and forced dialysis. The value obtained was $\gamma = 11 \pm 1$.

Sources—ATP, AMP-PNP, and AMP-PCP were from Boehringer Mannheim. ADP, AMP, CTP, UTP, and crystalline bovine serum albumin were from Sigma and TNP-ADP were from Molecular Probes, Inc. of Plano, TX. 2,4,6-Trinitrobenzenesulfonic acid was from Eastman. [2,8-3H]ATP and [3H]ouabain were from New England Nuclear and Amersham, respectively.

RESULTS

Titrations of Na,K-ATPase with TNP-ATP

Fig. 2 shows a typical TNP-ATP fluorescence titration in the presence and absence of enzyme. In this experiment, consecutive TNP-ATP additions were made to the same cuvette and the total fluorescence intensity after each addition was plotted as a function of the total TNP-ATP concentration. In the presence of (Na,K)-ATPase, a high affinity fluorescence enhancement was observed. This enhancement appears to saturate since the slope of the plot of fluorescence versus TNP-ATP concentration in the presence of enzyme approaches that in buffer alone. The fluorescence enhancement was prevented or reversed by addition of 2-4 mM ATP, suggesting it reflects TNP-ATP binding to a nucleotide site as discussed in the next section.

In the absence of ions that are ligands of the enzyme, such as Na\(^+\), K\(^+\), and Mg\(^{2+}\), TNP-ATP fluorescence in the presence of (Na,K)-ATPase was constant and stable for at least 1 h. Under these conditions, the interaction of TNP-ATP with the enzyme was at apparent equilibrium and fluorescence titrations as in Fig. 2 were used to determine its equilibrium-binding parameters. However, the fluorescence method can provide information only on binding sites that enhance TNP-ATP fluorescence. TNP-ATP binding to sites without fluorescence enhancement would be undetectable by this method.

As an alternative approach, we synthesized [3H]TNP-ATP and compared the binding data obtained by forced dialysis with those from fluorescence measurements. Fig. 3 shows Scatchard plots derived from two fluorescence titrations at different temperatures using nonradioactive TNP-ATP, one fluorescence titration with [3H]TNP-ATP, and one radioactive titration of [3H]TNP-ATP measured by forced dialysis, all with the same enzyme preparation. Although TNP-ATP binding data measured by forced dialysis exhibited more scatter than by fluorescence titration, both methods gave similar results. This indicates that the fluorescence data are a reliable measure of TNP-ATP binding and that significant binding without fluorescence enhancement did not occur.

The linear plots in Fig. 3 indicate that TNP-ATP binds with high affinity to a homogeneous population of sites. Table I summarizes the values for the dissociation constant and number of binding sites in several (Na,K)-ATPase preparations at different temperatures, obtained with either fluorescence or radioactivity measurements. The $K_D$ values for TNP-ATP binding ranged from 35-90 nM at 3°C and from 0.21-0.71 $\mu$M at 23 ± 2°C. We suspect that the variability in the $K_D$ values is due to the presence of contaminating ligands (such as ATP, ADP, or NH\(_4\)^+) that were introduced in the enzyme preparation during the purification (31). In support of this, we have observed that the higher $K_D$ values were obtained when higher enzyme concentrations were used. Furthermore, measurements of antihyolubain binding rate in the absence of added ATP and in the presence of an ATP-regenerating system show significant rates, indicating some contamination of the enzyme with either ATP or ADP (35).

The number of TNP-ATP binding sites ranged from 3.39-6.01 nmol/mg of protein in several preparations when enzyme protein was determined by amino acid analysis and TNP-ATP binding was measured by either fluorescence or radioactivity (Table I). The number of [3H]ouabain binding sites was 5.09 ± 0.16 and 5.2 ± 0.2 nmol/mg in two different preparations, also based on protein determined by amino acid analysis. The ratio of the average number of ouabain sites to TNP-ATP sites was 0.91. This indicates that there is one TNP-ATP site/ouabain site in (Na,K)-ATPase.

If instead of using amino acid analysis the protein was determined by the Lowry (36) method and the concentration of the albumin standard was determined either gravimetrically or by absorbance at 280 nm, the maximum number of TNP-ATP binding sites was in the range of 3.8-4.3 nmol/mg, as previously reported by this laboratory (33, 35) and as exemplified by the Scatchard plots in Fig. 6. Therefore, in our hands, the Lowry assay overestimates (Na,K)-ATPase protein concentration by a factor of 1.28-1.41 when based on gravimetric or 280-nm absorbance determinations of the albumin standard or by a factor of 1.07-1.18 when albumin concentration is determined by amino acid analysis. It is noteworthy that the Lowry assay also overestimates the protein in (Ca)-ATPase preparations by 20% when compared to dry weight measurements (46).
ATP, as measured by fluorescence. This binding was insensitive to the above stoichiometries. If the enzyme preparation contained a large number of low affinity sites, nucleotides competed with TNP-ATP binding with the apparent Kd, for TNP-ATP 6-fold, while a 100-fold higher AMP concentration increased Kd less than 2-fold.

The Kd for various nucleotides was calculated from experiments such as those shown in Fig. 4, assuming binding competition at a single site. The Kd values in Table II indicate that nucleotides competed with TNP-ATP binding with the following sequence of decreasing affinity: ATP > ADP > AMP-PNP > AMP-PCP > CTP ≈ UTP ≈ AMP. The Kf values obtained with two different competitor concentrations (Table II) were in agreement for most of the nucleotides tested, indicating that the single site treatment is valid. AMP and UTP, however, did not exhibit true competitive behavior since a 10-fold increase in concentration did not increase the measured Kapp by a factor of 1 + [I]/Kd. AMP and UTP appear to bind poorly, if at all, as previously concluded from ATP-binding data (1, 2).

**Effect of Na+ and K+ on TNP-ATP Binding**

It has been previously established that ATP, ADP, and AMP exhibit cation-sensitive fluorescence enhancement when TNP-ATP was added to sonicated egg lecithin liposomes. We interpret these observations as evidence of low affinity electrostatic binding of TNP-ATP to phospholipids. Since the nonspecific sites had much lower affinity, they would have shown up as a curvature near saturation in the Scatchard plot. This was not seen in the absence of ligands (Fig. 3), but was seen sometimes at high enzyme and TNP-ATP concentrations, particularly when NaCl or MgCl2 was present (e.g. Fig. 6). The value of fluorescence enhancement upon TNP-ATP binding to nonspecific sites is not known; therefore, the actual magnitude of the binding to these sites cannot be quantitated. If we assume that TNP-ATP bound to the nonspecific sites has the same quantum yield as in the high affinity sites, fluorescence measurements in the absence of 2-4 mM ATP at high (Na,K)-ATPase concentration suggest that a maximum of about 5% of TNP-ATP binding at saturation in the experiments of Table I could have been due to low affinity sites.

**Effect of Nucleotides on TNP-ATP Binding**

The nucleotide specificity of the TNP-ATP binding site was investigated by TNP-ATP titrations in the presence of various nucleotides. Fig. 4 shows representative Scatchard plots of TNP-ATP binding derived from fluorescence titrations in the absence and presence of ATP and AMP. Although the number of sites remained essentially constant, 3.9 μM ATP increased the apparent Kd for TNP-ATP 6-fold, while a 100-fold higher AMP concentration increased Kd less than 2-fold.

The Kd for various nucleotides was calculated from experiments such as those shown in Fig. 4, assuming binding competition at a single site. The Kd values in Table II indicate that nucleotides competed with TNP-ATP binding with the following sequence of decreasing affinity: ATP > ADP > AMP-PNP > AMP-PCP > CTP ≈ UTP ≈ AMP. The Kf values obtained with two different competitor concentrations (Table II) were in agreement for most of the nucleotides tested, indicating that the single site treatment is valid. AMP and UTP, however, did not exhibit true competitive behavior since a 10-fold increase in concentration did not increase the measured Kapp by a factor of 1 + [I]/Kd. AMP and UTP appear to bind poorly, if at all, as previously concluded from ATP-binding data (1, 2).

**Table I**

| Preparation | Method | Temperature °C | Kd μM | n nmol/mg |
|-------------|--------|----------------|-------|-----------|
| 1           | Fluorescence | 23 | 0.54 |
| 2           | Fluorescence | 25 | 0.35 | 5.83 ± 0.06 |
| 3           | Fluorescence | 25 | 0.35 | 6.01 ± 0.10 |
| 4           | Fluorescence | 25 | 0.35 | 5.44 ± 0.07 |
| 5           | Fluorescence | 3 | 0.053 | 5.57 ± 0.03 |
| 6           | Fluorescence | 3 | 0.035 | 5.77 ± 0.08 |
| 7           | Fluorescence | 3 | 0.077 |
| 8           | Fluorescence | 3 | 0.082 |
| 9           | Fluorescence | 3 | 0.090 |
| 10          | Fluorescence | 22 ± 2 | 0.40 |
| 11          | Fluorescence | 22 ± 2 | 0.21 | 5.80 ± 0.38 |
| 12          | Forced dialysis | 22 ± 2 | 0.71 | 5.39 ± 0.19 |
| 13          | Forced dialysis | 22 ± 2 | 0.27 | 5.66 ± 0.21 |

*E. G. Moczydlowski, unpublished results.*

**Fig. 3.** Scatchard plots of TNP-ATP binding to eel (Na,K)-ATPase. The measurement of bound and free TNP-ATP was made directly by the forced dialysis method using [3H]TNP-ATP (○) or by fluorescence titration with [3H]TNP-ATP (×) or unlabeled TNP-ATP (■, △). The same enzyme preparation, No. 3 of Table I, was used in each of the four titrations and the protein concentration was determined by amino acid analysis. The buffer was approximately 45 mM Tris-HCl, pH 7.5, and 1 mM CDTA or EDTA in each experiment and individual protein concentrations and temperatures are as follows: 59 μg/ml, 3°C (■); 45 μg/ml, 25°C ( ), 48 μg/ml, 21°C ( ×); and 177 μg/ml, 22°C ( ○). The lines drawn through the data were calculated by least squares analysis. The dashed line corresponds to the forced dialysis method (○) and the solid lines correspond to the respective fluorescence experiments. The derived values for n and Kd are shown in Table I.

Based on the number of binding sites in Table I, the maximum molecular weight/TNP-ATP binding site ranged from 1.64 × 10^6 to 1.92 × 10^6 with an average value of 1.75 × 10^6. Similarly, the maximum molecular weight/ouabain binding site was (1.94 ± 0.02) × 10^6. These values are upper limits since the presence of either denatured (Na,K)-ATPase that did not bind these ligands, or extraneous protein due to incomplete purification, or both would lead to underestimation of the binding capacity. It is unlikely that nonspecific adsorption of TNP-ATP to lipid or protein sites contributed significantly to the above stoichiometries. If the enzyme preparation contained a large number of low affinity sites in which TNP-ATP would partition, complete displacement of radioactive TNP-ATP by cold TNP-ATP should not be observed. When a 200-fold excess of cold TNP-ATP was added to [3H]TNP-ATP bound near saturating conditions (5 nmol/mg), more than 99% of the radioactive bound label was released, as measured by forced dialysis, indicating negligible nonspecific binding. However, in fluorescence titrations at high enzyme concentrations (~1 mg/ml), the slope of the fluorescence versus concentration plot at TNP-ATP concentrations well beyond saturation of the high affinity enhancement was larger than that in buffer alone, suggesting the existence of low affinity binding sites. TNP-ATP binding to these sites showed no evidence of saturation up to 50 μM TNP-ATP, as measured by fluorescence. This binding was insensitive to 4 mM ATP and increased with divalent cation concentration. A similar low affinity, divalent cation-sensitive fluorescence enhancement was observed when TNP-ATP was added to sonicated egg lecithin liposomes. We interpret these observations as evidence of low affinity electrostatic binding of TNP-ATP to phospholipids. Since the nonspecific sites had much lower affinity, they would have shown up as a curvature near saturation in the Scatchard plot. This was not seen in the absence of ligands (Fig. 3), but was seen sometimes at high enzyme and TNP-ATP concentrations, particularly when NaCl or MgCl2 was present (e.g. Fig. 6). The value of fluorescence enhancement upon TNP-ATP binding to nonspecific sites is not known; therefore, the actual magnitude of the binding to these sites cannot be quantitated. If we assume that TNP-ATP bound to the nonspecific sites has the same quantum yield as in the high affinity sites, fluorescence measurements in the absence of 2-4 mM ATP at high (Na,K)-ATPase concentration suggest that a maximum of about 5% of TNP-ATP binding at saturation in the experiments of Table I could have been due to low affinity sites.

**Effect of Nucleotides on TNP-ATP Binding**

The nucleotide specificity of the TNP-ATP binding site was investigated by TNP-ATP titrations in the presence of various nucleotides. Fig. 4 shows representative Scatchard plots of TNP-ATP binding derived from fluorescence titrations in the absence and presence of ATP and AMP. Although the number of sites remained essentially constant, 3.9 μM ATP increased the apparent Kd for TNP-ATP 6-fold, while a 100-fold higher AMP concentration increased Kd less than 2-fold.

The Kd for various nucleotides was calculated from experiments such as those shown in Fig. 4, assuming binding competition at a single site. The Kd values in Table II indicate that nucleotides competed with TNP-ATP binding with the following sequence of decreasing affinity: ATP > ADP > AMP-PNP > AMP-PCP > CTP ≈ UTP ≈ AMP. The Kf values obtained with two different competitor concentrations (Table II) were in agreement for most of the nucleotides tested, indicating that the single site treatment is valid. AMP and UTP, however, did not exhibit true competitive behavior since a 10-fold increase in concentration did not increase the measured Kapp by a factor of 1 + [I]/Kd. AMP and UTP appear to bind poorly, if at all, as previously concluded from ATP-binding data (1, 2).

**Effect of Na+ and K+ on TNP-ATP Binding**

It has been previously established that ATP, ADP, and
ATP binding was measured in these and similar experiments are listed in Table I. 

Fluorescence decrease was sigmoid with Hill coefficient, $n = 3.9$ under "Experimental Procedures." Conditions: 50 mM Hepes-Tris, pH 7.5, 22 mM NaCl, 0.8 mM EDTA, 70 ng/ml of eel (Na,K)-ATPase, 3.9 μM ATP (C), and 0.39 mM AMP (X). Temperature = 3°C. The $K_D$ values for TNP-ATP and several different nucleotides derived from these and similar experiments are listed in Table II.

Formycin triphosphate binding to (Na,K)-ATPase is antagonized by K" and that the effect of K" is reversed by Na" (1, 7, 8, 47, 48). TNP-ATP binding also exhibited this sensitivity to monovalent cations. If K" was added to a cuvette containing TNP-ATP and (Na,K)-ATPase, a rapid fluorescence decrease was observed. If sufficient Na" was subsequently added, there was a rapid fluorescence increase and complete reversal of the K" effect. These changes appear to reflect K"-promoted dissociation and Na"-promoted binding of TNP-ATP. The rapid fluorescence changes reached a steady state within 5 s after the ion addition with no further fluorescence change at low temperature (0-3°C). At 25°C, slow fluorescence increases ($t_{1/2} = 10$ min) followed the fast change, particularly after K" addition, but were also observed with Na", as discussed in a later section. However, if consecutive additions of Na" or K" were made about once every 20 s, it was possible to titrate the fast fluorescence change with little contribution from the slow component. Fig. 5A shows the dependence of the fast fluorescence decrease as a function of K" concentration in the absence and presence of Na". In the absence of Na", the fluorescence decrease was half-maximal at 0.5 mM K", with Hill coefficient, $n = 1.1$. In the presence of 27 mM Na", the fluorescence decrease was sigmoid with $K_{0.5} = 32$ mM and $n = 1.8$. Similarly, Fig. 5B shows the fluorescence increase as a function of Na" concentration in the presence of 27 and 54 mM K". Both curves are sigmoid with $n = 2$ and $K_{0.5} = 19$ and 30 mM, respectively. This behavior suggests that the effects of these ions on TNP-ATP fluorescence reflect changes in TNP-ATP binding that result from specific interactions of Na" and K" with their binding sites on the enzyme. The increases in $K_{0.5}$ values demonstrate the antagonistic consequences of Na" or K" binding.

The following observations suggest that the rapid changes in TNP-ATP fluorescence caused by Na" and K" primarily

---

**Table II**

Nucleotide specificity of the TNP-ATP binding site

$K_D$ for the nucleotides was measured from TNP-ATP fluorescence titrations in the absence and presence of two different competitor concentrations, as described in the text and the legend to Fig. 4. The average $K_D$ and the range for the two measurements are listed.

| Ligand   | $K_D$ (μM) |
|----------|------------|
| TNP-ATP  | 0.08 ± 0.003 |
| ATP      | 1.76 ± 0.03  |
| ADP      | 1.6 ± 0.2    |
| AMP-PNP  | 3.8 ± 1.1    |
| AMP-PNP  | 22 ± 0.2     |
| CTP      | 81 ± 15      |
| AMP      | >1000        |
| UTP      | >1000        |

---

**Fig. 4.** Effect of ATP and AMP on TNP-ATP binding. TNP-ATP binding was measured by fluorescence titration as described under "Experimental Procedures." Conditions: 45 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 91 ng/ml of eel (Na,K)-ATPase, 2.3 μM TNP-ATP, and the indicated concentrations of NaCl or KCl. Temperature = 25°C. Fluorescence is expressed as a ratio of the observed fluorescence to the initial fluorescence and was measured as described under "Experimental Procedures." The respective $K_{0.5}$ values for KCl and NaCl and the Hill coefficients (n) derived from Hill plots of the data are: 0.5 mM KCl, 1.1 (○); 27 mM KCl, 1.8, (×); 19 mM NaCl, 2.0 (●); and 30 mM NaCl, 2.0 (△).

**Fig. 5.** Effect of Na" and K" on TNP-ATP fluorescence in the presence of (Na,K)-ATPase. The rapid fluorescence decrease observed when KCl was added to enzyme and TNP-ATP is plotted in A. The rapid fluorescence increase observed when NaCl was added to enzyme plus TNP-ATP and KCl is plotted in B. Conditions: 45 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 70 ng/ml of eel (Na,K)-ATPase, 2.3 μM TNP-ATP, and the indicated concentrations of NaCl or KCl. Temperature = 25°C. Fluorescence is expressed as a ratio of the observed fluorescence to the initial fluorescence and was measured as described under "Experimental Procedures." The respective $K_{0.5}$ values for KCl and NaCl and the Hill coefficients (n) derived from Hill plots of the data are: 0.5 mM KCl, 1.1 (○); 27 mM KCl, 1.8, (×); 19 mM NaCl, 2.0 (●); and 30 mM NaCl, 2.0 (△).
When (Na,K)-ATPase was titrated at 0-3°C with TNP-ATP it reflects changes in TNP-ATP affinity, not quantum yield. When (Na,K)-ATPase was titrated at 0-3°C with TNP-ATP in the presence of 3-20 mM KCl, the maximum fluorescence enhancement was the same as in the absence of ionic ligands, although higher TNP-ATP concentrations than in the absence of K+ were necessary to reach saturation. In contrast, NaCl had little effect on either the concentration dependence or the maximum enhancement (not shown).

Fig. 6 shows Scatchard plots of TNP-ATP binding at 3°C in the presence of 20 mM NaCl or KCl derived from fluorescence data assuming no difference in quantum yield in the presence of these ions. The KD for TNP-ATP increased 3-fold in the presence of Na+ relative to Na- with no change in the number of binding sites. Fig. 6 also shows the contribution of low affinity sites, discussed above. In a separate experiment, titrations with different K+ concentrations showed that the KD for TNP-ATP increased from 0.09 μM in the absence of ions to 0.38 μM with 3 mM K+ with no further increase up to 12 mM K+ (not shown). A similar effect of K+ on the KD of ATP has been reported (47).

Effect of Ions on Displacement of TNP-ATP by ATP

Competition between TNP-ATP and ATP for binding was also demonstrated by adding increasing concentrations of ATP to a cuvette containing TNP-ATP plus enzyme and measuring the fluorescence decrease due to displacement of TNP-ATP from its binding site. Experiments of this type were used to measure the effect of various ligands on ATP binding to the TNP-ATP site.

Fig. 7A shows a series of such displacement titrations at four TNP-ATP concentrations. The ordinate axis is a measure of the fraction of TNP-ATP bound relative to the amount in the presence of 3-20 mM KCl, the maximum fluorescence enhancement was the same as in the absence of ionic ligands. In contrast, NaCl had little effect on either the concentration dependence or the maximum enhancement (not shown).

Fig. 7B shows three separate displacement titrations under three different ligand conditions with the same amount of ATP and enzyme. The ligand combinations shown (Na+, K+, and K+ + Mg2+) are ones which produce minimal ATP hydrolysis at 3°C. With them the TNP-ATP fluorescence signal was stable with time in the presence of enzyme. Fig. 7B shows that the various ligands affected the ability of ATP to displace TNP-ATP.

Assuming that ATP (S) and TNP-ATP (I) bind to a single homogeneous site (E), the mass action expressions for binding of S and I, equal to their respective dissociation constants, Ks and Ki, may be substituted into the total enzyme conservation equation and rearranged to give:

\[ [I] \frac{[E]}{[E]_0} - 1 = K_s \left( 1 + \frac{[S]}{K_s} \right) \]

where [E] is the concentration of total enzyme and [I], [E], and [S] are the concentrations of free and bound TNP-ATP and free ATP, respectively. A plot of the term on the left versus [S] yields a straight line with the ordinate intercept equal to Ks and the abscissa intercept equal to -Ks. The fluorescence enhancement from experiments such as those shown in Fig. 7, A and B, was used to estimate [E] and from that [I] in Equation 5. The total concentration of ATP added was taken to be the concentration of free ATP since the total enzyme concentration was relatively insignificant. In the presence of Mg2+ (Fig. 7B), more than 90% of the total ATP existed as MgATP since the stability constant for MgATP is 3 × 10^4 (24). When the data were plotted according to Equa-

![Fig. 6. Scatchard plots of TNP-ATP binding in the presence of Na+ or K+. TNP-ATP binding was measured by fluorescence titration at 3°C. The cuvettes contained 50 mM Tris-HCl, pH 7.5, 0.15 mM CDTA, 80 μg/ml of eel (Na,K)-ATPase, and 20 mM NaCl or 20 mM KCl. The protein concentration in this experiment is based on the Lowry (36) assay with a bovine serum albumin standard determined by absorbance.](image)

![Fig. 7. Effect of Na+, K+, and Mg2+ on displacement by ATP of TNP-ATP bound to eel (Na,K)-ATPase. TNP-ATP was added to enzyme in the presence of no ligands, NaCl, KCl, or KCl + MgCl2. Tris-ATP was added at concentrations plotted along the abscissa and the decrease of TNP-ATP fluorescence was measured as described under "Experimental Procedures." In A and B, the ordinate is a measure of the fraction of TNP-ATP bound relative to the amount bound in the absence of ATP. The cuvettes contained 45 mM Tris-HCl, pH 7.5, and either 0.15 mM CDTA (no ligands), 20 mM NaCl + 0.15 mM CDTA, 20 mM KCl + 0.15 mM CDTA, or 20 mM KCl + 3.9 mM MgCl2 as indicated. Temperature = 3°C. In A, four separate titrations are shown at constant enzyme (45 μg/ml) and increasing [TNP-ATP] as indicated. In B, the cuvettes contained 67 μg/ml of enzyme, 1.33 μM TNP-ATP, and various ligands as indicated.](image)
tion 5, straight lines were obtained (not shown) with correlation coefficients greater than 0.99. This fit between the data and the equation is consistent with competition between TNP-ATP and ATP at a single site.

For each ligand condition, ATP displacement titrations were performed at four TNP-ATP concentrations. The data were analyzed and plotted according to Equation 5 and \( K_a \) and \( K_t \) were calculated by least square analysis of the individual titrations or of data from all four titrations pooled together. The derived dissociation constants for ATP and TNP-ATP are presented in Table III. The two averaging methods gave similar results. Table III shows that the affinity for TNP-ATP remained high under all ligand conditions, whereas the affinity for ATP decreased more than 10-fold in the presence of K\(^+\) alone and of the order of a 100-fold when both Mg\(^{2+}\) and K\(^+\) were added. Another persistence of a high affinity for TNP-ATP, under conditions that greatly reduced the affinity for ATP, was demonstrated also by our finding that TNP-ATP bound to the ouabain-enzyme complex with a \( K_a \) of about 1.2 \( \mu M \) at 0°C and that ATP concentrations higher than 1 mM inhibits ATP binding (1, 49). Studies of the interaction of TNP-ATP with the ouabain-complexed enzyme will be reported elsewhere.

**Slow TNP-ATP Fluorescence Changes in the Presence of (Na,K)-ATPase**

Under certain incubation conditions, the fluorescence changes after mixing TNP-ATP with enzyme exhibited complex kinetics in the sense that an initial rapid fluorescence increase was followed by slow fluorescence changes. At 23°C, slow increases in fluorescence with a half-time of about 10 min were observed after an initial rapid rise when K\(^+\) or NH\(_4\)\(^+\) was included in the incubation medium or when high enzyme concentrations (>0.2 mg/ml) in the absence of added ligands were used. The magnitude of these slow changes varied between 5 and 30% of the total fluorescence change in different preparations and they were greatly reduced or absent at low temperature (0-3°C) or low enzyme concentrations (<0.5 \( \mu g/\)ml). This behavior does not appear to be related to permeability barriers such as those of closed vesicles since addition of sufficient Lubrol (1%) to solubilize the enzyme had no effect on the rate nor extent of these slow fluorescence changes.

**TABLE III**

Effect of ions on the affinity of (Na,K)-ATPase for ATP and TNP-ATP

| Added ligands | \( K_a \) ATP \( \mu M \) | \( K_a \) TNP-ATP \( \mu M \) |
|---------------|--------------------------|-----------------------------|
| None          | 1.1 ± 0.7                | 0.04 ± 0.02                 |
| NaCl          | 1.0 ± 0.9                | 0.05 ± 0.04                 |
| KCl           | 3.9 ± 0.8                | 0.23 ± 0.07                 |
| KCl + MgCl\(_2\) | 18.6 ± 2.5       | 0.37 ± 0.04                 |
| KCl           | 14.7 ± 5.5               | 0.41 ± 0.11                 |
| KCl + MgCl\(_2\) | 122 ± 35                | 0.21 ± 0.07                 |
| KCl + MgCl\(_2\) | 71 ± 20                 | 0.14 ± 0.05                 |

These phenomena may reflect slow conformational changes in the enzyme that affect TNP-ATP binding, or the quantum yield of bound TNP-ATP, or both and may contribute to the larger scatter observed in the Scatchard plot data at 23°C compared to those at 0-3°C (Fig. 0).

**Effect of Mg\(^{2+}\)**—When TNP-ATP was added to enzyme in the presence of MgCl\(_2\), the rapid fluorescence increase was followed by a slow decrease in fluorescence which was dependent on the concentration of TNP-ATP. Fig. 8 shows superimposed chart traces from five different runs in which varying amounts of TNP-ATP were added to a cuvette containing 219 \( \mu g/ml \) of eel (Na,K)-ATPase and 4.7 mM MgCl\(_2\) at 25°C. At 0.45 and 0.67 \( \mu M \) TNP-ATP, the rapid initial fluorescence increase was followed by a small increase for about 10 min and then it remained essentially constant for 50 min. However, during the following 1-2 h, a fluorescence decrease was observed (not shown). This decrease occurred without a lag at 0.9, 1.35, and 2.69 \( \mu M \) TNP-ATP, as shown in Fig. 8.

The absolute magnitude of the fluorescence decrease appeared to be constant over the range of TNP-ATP concentration examined, 0.9-5.4 \( \mu M \). Although the fluorescence appeared to decrease to an equilibrium level in Fig. 8, the system was not stable. If the fluorescence was measured for several hours longer, a slower increase with a half-time of 1 h or longer followed the apparent equilibrium.

Addition of sufficient EDTA to chelate all of the Mg\(^{2+}\) prevented the fluorescence decrease when it was added initially or stopped the fluorescence decrease when it was added at any subsequent time. However, no reversal of the fluorescence decrease was observed after EDTA addition once the decrease had occurred.

The slow fluorescence decrease cannot be ascribed to TNP-ATP hydrolysis or enzyme phosphorylation, since TNP-ADP produced the same response. The slow fluorescence change must have been induced by the binding of the TNP-nucleotide since preincubation of the enzyme for various lengths of time with Mg\(^{2+}\) did not alter the time course of the change. Also, replacement of Mg\(^{2+}\) by Mn\(^{2+}\) or Co\(^{2+}\) produced similar slow fluorescence decreases. The apparent half-time of the Mg\(^{2+}\)-dependent fluorescence decrease changed from 16 min at 24°C to 4.5 min at 38°C. At 0-3°C, the decrease was much slower, and addition of Na\(^+\) or K\(^+\) appeared to suppress the phenomenon. However, the addition of Na\(^+\) or K\(^+\) at room tempera-
ture had little effect on the kinetics of the Mg\(^{2+}\)-induced fluorescence decrease.

Fluorescence excitation and emission spectra taken before and after the Mg\(^{2+}\)-induced decrease revealed no significant spectral shape changes except a slight broadening of the emission spectrum. These results suggest that the decrease is not due to the formation of a different fluorescent species. An absorption spectrum taken after the Mg\(^{2+}\)-induced decrease and subsequent solubilization of the enzyme with SDS was identical with a TNP-ATP spectrum in the absence of enzyme, indicating that the fluorescence decrease is not the result of an irreversible chemical reaction with the enzyme which changes the spectral properties of the trinitro cyclohexadienate chromophore. Preliminary experiments in which the fraction of \([3H]\)TNP-ATP bound was determined by forced dialysis before and after the Mg\(^{2+}\)-induced fluorescence decrease had stabilized suggest that the slow fluorescence decrease corresponds to both TNP-ATP dissociation and a lower quantum yield of the bound probe.

Fluorescence Properties of TNP-ATP

We studied the spectroscopic parameters of TNP-ATP bound to (Na,K)-ATPase and in certain solvents in order to compare the properties of the (Na,K)-ATPase binding site with those of known environments. Fig. 9 shows corrected emission spectra of TNP-ATP in various solvents. There was a fluorescence enhancement and a blue shift of the emission maximum in solvents of lower polarity (dielectric constants between 24 and 36) than that of water (dielectric constant = 78) in agreement with Hiratsuka’s (50) observations in ethanol/water mixtures. In glycerol, which is more polar (dielectric constant = 42.5), but viscous (1490 centipoise) compared to the other solvents (0.6-1.2 centipoise), there was a large fluorescence enhancement but a smaller spectral shift, suggesting that TNP-ATP fluorescence is also sensitive to viscosity. The absorbance at 410 nm varied little in the different solvents, indicating that the fluorescence changes were mainly due to changes in quantum yield. The relative quantum yields with respect to that in water (=1) were: methanol (7.5), ethanol (12.2), glycerol (45.4), and dimethylformamide (45.8). The absolute quantum yield in dimethylformamide was 0.01. The absorption and fluorescence emission properties of TNP-ADP and TNP-AMP were similar to those of TNP-ATP; this similarity implies that the β and γ phosphates have little effect on the chromophore.

Fig. 10 shows the absorption spectrum of TNP-ATP in 50 mM Tris-HCl, pH 7.5, and excitation polarization spectra in buffer and ethanol. The change in steady state anisotropy from positive values at 500-520 nm to negative values at 380-420 nm indicates that the short and long wavelength absorption bands correspond to separate oscillators with transition moments oriented at an angle to each other. The polarization of TNP-ATP in glycerol did not vary with temperature in the range of 6-30°C and was identical with that measured in a rigid glass of propylene glycol at -50°C (Fig. 10), indicating that the polarization is independent of molecular motion under these conditions, i.e. is equivalent to the limiting anisotropy, rₒ. The measured value of rₒ = -0.12 at 408 nm gives an angle, θ, of 68.6° between the absorption transition moment of the short wavelength oscillator and the emission transition moment, as calculated from (51):

\[
rₒ = \frac{3 \cos^2 \theta - 1}{5}
\]

At 510 nm, rₒ = 0.34, which is slightly less than the theoretical limit of 0.4 expected for strict co-linearity of the lowest lying absorption and the emission oscillators.

Fig. 10 also shows that the polarization was high in both glycerol and water; consequently, it was rather insensitive to solvent viscosity. This indicates a very short fluorescence lifetime (~10⁻¹¹ s) of the probe in water. In ethanol and dimethylformamide, partial depolarization was observed, indicating a longer fluorescence lifetime during which molecular rotation occurs. The rotational correlation time of TNP-ATP should be about 0.15 ns in ethanol at 25°C, as calculated from

![Fig. 9. Emission spectra of 8.3 μM TNP-ATP in various solvents. Samples were in 3-mm cuvettes. The spectrum labeled “H₂O” contained 50 mM Tris-HCl, pH 7.5. DMFA is dimethylformamide. λₑ = 410 nm. Temperature = 25°C.](image)

![Fig. 10. Absorption and polarization spectra of TNP-ATP. The trace with maxima at 408 nm and 470 nm is the absorption spectrum in H₂O. The other traces show the anisotropy values in the indicated solvents. For the polarization measurements, fluorescence emission was measured with a Corning 3-98 cut-off filter placed on the emission side. Slits = 4-nm excitation and 20-nm emission. The samples contained 10 μM TNP-ATP. Anisotropy was measured as described under “Experimental Procedures.” The measurements in ethanol and dimethylformamide (DMFA) were taken at 25°C. The spectrum labeled “H₂O” contained 50 mM Hepes-Tris, pH 7.5, and was taken at 0°C. The polarization in glycerol was constant over the temperature range 6-30°C.](image)
The Nature and Properties of the TNP-ATP Binding Site

TNP-ATP binds to a nucleotide site of (Na,K)-ATPase with high affinity and specificity. Several lines of evidence indicate that this site is the high affinity catalytic site of the enzyme for ATP. 1) There was one homogeneous TNP-ATP site/ouabain site (Fig. 3 and Table I); 2) ATP and other nucleotides competed with TNP-ATP for binding (Fig. 4 and Table II) with the same affinity and nucleotide specificity previously shown for ATP binding (1, 2); 3) K+ decreased the affinity for TNP-ATP and Na+ antagonized the K+ effect (Figs. 5 and 6) as previously described for ATP (1, 47, 48), ADP (55), and formycin triphosphate (7, 8); 4) TNP-ATP inhibited competitively with respect to ATP both Na+-activated ATP hydrolysis and enzyme phosphorylation with derived $K_I$ values similar to the $K_D$ determined by direct binding (27). The only alternative interpretation of these data we can think of is that TNP-ATP binds to a site that is allosterically regulated by ATP binding to the substrate site; i.e. that the TNP-ATP and ATP sites are different but their occupancy is mutually exclusive. We consider this unlikely. Despite the similarities noted in ATP and TNP-ATP binding to (Na,K)-ATPase, certain properties of the interaction of the enzyme with TNP-ATP are different. The chromophore increased the binding energy of the nucleotide as indicated by the higher affinity of TNP-ATP over that of ATP. TNP-ADP had a higher affinity than TNP-ATP and even TNP-AMP binds significantly since it inhibits (Na,K)-ATPase at concentrations above 10 $\mu$M (27), whereas AMP binds poorly (Table II). The increased affinity of the TNP-nucleotides may result from electrostatic interactions of the negative charge on the chromophore with a cationic residue, such as arginine (56, 57), at or near the location of ribose in the ATP site. Hydrophobic interactions of the chromophore at the active site may contribute additional binding energy. The presence of the chromophore may alter the positioning of the nucleotide or its $\beta - \gamma$ phosphate bond in the catalytic center since TNP-ATP is not hydrolyzed by (Na,K)-ATPase (27), although it is a good substrate for myosin (25).

Of particular interest is the finding that (Na,K)-ATPase retained a high affinity ($K_D = 0.1-1$ $\mu$M) for TNP-ATP and TNP-ADP in the presence of ligands, such as Mg$^{2+}$ and K+ or ouabain, that cause large decreases in affinity for ATP. This property explains the inhibitory potency of the TNP-nucleotides (27) and allowed fluorimetric measurements of ATP binding, by competition with TNP-ATP, even when the affinity for ATP was low, as discussed below.

We must point out, however, that the slow TNP-ATP fluorescence increase in the presence of monovalent cations and the slow TNP-ATP fluorescence decrease in the presence of Mg$^{2+}$ (Fig. 8) remain unexplained. These phenomena were readily detectable above 20°C, but were either absent or insignificantly slow at low temperature (0–3°C). It is probable that they reflect slow conformational changes induced by TNP-ATP binding in the presence of certain ionic ligands, causing either changes in TNP-ATP affinity or quantum yield or both. When the slow changes are present, the interpretation of TNP-ATP fluorescence decreases upon ATP addition is complex, since the system is not at equilibrium and factors other than simple displacement of TNP-ATP from the enzyme may contribute to the observed signal. Therefore, the present studies of the effect of Na+, K+, and Mg$^{2+}$ on competition between TNP-ATP and ATP were carried out at low temperature, where TNP-ATP fluorescence was stable. Further work should provide information on the nature of the slow fluorescence changes. This may allow proper design and
interpretation of experiments in which TNP-ATP fluorescence is used to study ATP binding at the higher temperatures usually used to study enzyme turnover.

In addition to the usefulness of TNP-ATP to study ATP binding, TNP-ATP and TNP-ADP may have wide application in energy transfer studies to investigate the structure of (Na,K)-ATPase. Although the relatively low quantum yield and short lifetime of TNP-ATP preclude its use as an energy transfer donor, it is an excellent acceptor for blue emitting fluorophores. It has strong absorbance in the 390–520-nm region consisting of two oscillators with their transition moments at an angle of 69° to each other and appears to exhibit some mobility in its binding site. These properties tend to reduce the uncertainties due to fixed orientations of the probes in distance estimates from energy transfer measurements (54).

**Does (Na,K)-ATPase Exhibit “Half-of-Sites” Reactivity?**

Although it is generally accepted that (Na,K)-ATPase displays “half-of-sites” behavior toward phosphorylation and ATP or ouabain binding (3, 17–22), rigorous experimental support is lacking. Conclusive demonstration of this behavior requires accurate knowledge of the molecular weight, subunit composition, and maximum binding capacities. However, published values for these parameters are either controversial or subject to uncertainties. Radiation inactivation experiments gave an average molecular weight of 250,000, but the actual experimental values ranged from 190,000 to 500,000 (58, 59). Ultraceftinfractiug experiments have given molecular weights ranging from 276,000 (60) to 380,000 (61) for elasmobranch (Na,K)-ATPase. Based on SDS-polyacrylamide gel electrophoresis, the large (a) and small (b) polypeptides from various species have molecular weights of 90,000–100,000 and 30,000–60,000, respectively (14, 16, 18, 62, 63). By ultracentrifugation, the molecular weight of the a chain of elasmobranch (Na,K)-ATPase is 106,000 (61) and by gel filtration the molecular weight of dog kidney a chain is 121,000 (64). The molecular weight of the a chain may be underestimated by SDS gel electrophoresis owing to increased mobility of hydrophobic polypeptides in SDS gels compared to the water-soluble standards used to calibrate the gels (64, 65). Therefore, molecular weights determined by this method are lower limits. The eel (Na,K)-ATPase polypeptides migrate in SDS gels with apparent Mw of 96,500 (a) and 41,400 for the protein portion of b and are present in a 1:1 stoichiometry (39). This gives minimum molecular weights of 138,000 for an ab dimer and 276,000 for an ab2 tetramer. A previous study of highly purified eel (Na,K)-ATPase reported a maximum of 4.3 nmol/mg for ouabain binding and 4.36 nmol/mg for phosphorylation (16). These values correspond to a molecular weight for the binding unit of 229,000–233,000, which is lower than the minimum molecular weight of an ab2 tetramer. Any extraneous or denatured protein would lower the molecular weight of the binding unit further. These values are between the lowest estimates for the molecular weight of an ab dimer and an ab2 tetramer, so that “half-of-sites” behavior cannot be deduced from this information.

We measured TNP-ATP binding by either fluorescence or radioactivity using gel equilibration or forced dialysis. All three methods gave similar results. When the data were estimated using the Lowry protein determination, stoichiometries similar to those of ouabain, ATP, or phosphorylation, mentioned above, were obtained. However, when the protein was determined by amino acid analysis, we obtained an average of one TNP-ATP site/175,000 g of protein and one ouabain site/194,000 g of protein (Table I). The difference between the values for TNP-ATP and ouabain may be due to the presence of some radioactivity not associated with [3H]ouabain, since the [3H]ouabain was used as supplied and it has been shown that 5–10% of the radioactive material in similar [3H]ouabain preparations does not bind to (Na,K)-ATPase (49, 66).

Quantitative amino acid analysis is a direct method that does not involve assumptions about the extinction coefficient of proteins treated with the Folin phenol reagent, which varies up to 300% with different proteins (35). Since the Lowry (36) method has been used to determine protein in all of the binding studies mentioned above, it is possible that previously reported binding capacities have been underestimated. It is important to note that when protein was determined by amino acid analysis in a previous study of binding of a strophanthidin derivative to purified dog kidney (Na,K)-ATPase (67), the molecular weight of the binding unit was 175,000 ± 20,000, identical with our present results.

Our finding of one nucleotide site/175,000 g of protein (range 164,000–192,000) sets an upper limit for the protein molecular weight of the minimum protomer of the eel (Na,K)-ATPase. It is consistent with a protomer formed from one a and one b chain, but does not exclude the possibility that a small polypeptide of about 12,000 daltons (68, 69) is also a constituent, owing to the uncertainties in the molecular weights of the a and b polypeptides determined by SDS gel electrophoresis. Craig and Kyte (64) have recently reported that the molecular weight of an ab dimer in dog kidney (Na,K)-ATPase is 177,000 ± 13,000, in excellent agreement with our results.

One binding site/175,000 daltons is significantly less than the lowest estimate of 276,000 for the molecular weight of the protein portion of an ab2 oligomer in the eel enzyme. We conclude that (Na,K)-ATPase does not exhibit “half-of-sites” behavior.

**Effect of Ions on ATP Affinity**

Are “High” and “Low” Affinity ATP Sites Interconvertible?—On the basis of kinetic evidence, several workers have suggested that (Na,K)-ATPase has a high affinity catalytic site and a low affinity regulatory site for ATP (4–11, 13, 17, 20–24). Our results show that TNP-ATP binding and its competitive displacement by ATP can be quantitatively accounted for by a single noninteracting site with high TNP-ATP affinity that exhibits either high or low affinity for ATP, depending on which other ligands are bound to the enzyme (Fig. 7 and Table III). TNP-ATP inhibits ATPase activity competitively with ATP at the “high” affinity site in the presence of Na+ alone and competitively with ATP at the “low” affinity site in the presence of both Na+ and K+ (27). These results are consistent with the binding studies if a single ATP site changes from high to low affinity during turnover, as in the (Na,K)-ATPase mechanism discussed in the accompanying paper (27). The competition between ATP and TNP-ATP shows no evidence that high and low affinity ATP sites co-exist independently at low temperature. This suggests that the “high” and “low” affinity ATP “sites” observed kinetically reflect interconversion between two or more different conformations of a single site, rather than two structurally and functionally distinct sites.

**Acknowledgments**—We thank Jack Kyte for suggesting the determination of protein by amino acid analysis and assistance with the measurements, Rheto Strasser for help in measuring absorption spectra of TNP-ATP bound to (Na,K)-ATPase, Adriane Farkas for technical assistance, and Gregorio Weber and Warren Butler for the use of their instruments.

**REFERENCES**

1. Hegyvary, C., and Post, R. L. (1971) *J. Biol. Chem.* 246, 5234–5240.
