The Presence of Two Hydrolytic Sites on Beef Heart Mitochondrial Adenosine Triphosphatase*

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The ribose-modified nucleotides 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) and TNP-ADP were used to probe the catalytic sites on soluble beef heart mitochondrial adenosine triphosphatase (F1). Both compounds were potent competitive inhibitors of ATP hydrolysis catalyzed by F1, \( K_i = 5.5 \) and 10 nM, respectively, and by submitochondrial particles, \( K_i \) (TNP-ATP) = 21 nM. Both analogs inhibited the \( ^{32}P \)-ATP exchange reaction and the ATP-dependent reduction of NAD⁺ by succinate, catalyzed by submitochondrial particles.

TNP-ATP and TNP-ADP were bound by F1. The presence of two binding sites on the enzyme for TNP-adenine nucleotides was determined by titrations of difference absorbance spectra, of the increase in fluorescence of the analog which occurred upon interaction with protein, and by titrations with the centrifuge column method using \( ^{32}P \)-labeled TNP-adenine nucleotides. The first binding site bound the analogs with an affinity too high to be measured. The \( K_{in} \) for analog binding by the second site was 20 to 80 nM. In the presence of Mg²⁺, the 2 sites were filled with the TNP-ATP at a rate too rapid to be resolved by the procedure used.

TNP-[γ-\(^{32}P\)ATP was hydrolyzed by F1, \( K_m = 0.2 \) μM, \( V_{max} = 1.1 \) mol of \( ^{32}P \) formed/mol of F1/s. It was shown, using the isotope trap technique as well as the inhibitor efrapeptin, that the 2 binding sites for TNP-ATP on F1 are hydrolytic sites.

The molecular mechanism of action of energy-transducing ATPases constitutes one of the key elements in any understanding of energy conservation in the cell. For example, the mitochondrial ATPase catalyzes the terminal step in the synthesis of ATP from ADP and P, during oxidative phosphorylation. Although considerable insight has been gained into the properties of the molecule, many challenging questions remain which are directly relevant to mechanism. Among these is the number of catalytic sites on F1. In view of the presence of 2 to 3 copies of each of the major subunits in the molecule (1) and of multiple binding sites for adenine nucleotides (2, 3), it is probable that there is more than one catalytic site on the enzyme. In fact, a number of proposed mechanisms invoke 2 or more such sites (4-7). The most direct evidence regarding the number of catalytic sites available to date is provided by the report of Senior et al. (8) that a single gene codes for subunit \( \beta \) in the ATPase from Escherichia coli. Since it is probable that a hydrolytic site resides on subunit \( \beta \) (1), there should be as many hydrolytic sites in the molecule as \( \beta \) subunits.

This report describes the interactions of the adenine nucleotide analogs 2',3'-O-(2,4,6-trinitrophenyl)-ATP and TNP-ADP with beef heart mitochondrial ATPase. Earlier observations indicated that TNP-ATP was hydrolyzed by heavy meromyosin (9) and that both analogs were inhibitors of (Na⁺ + K⁺)-ATPases (10). Using the TNP-adenine nucleotides as probes of F1, we have found that the enzyme contains two high affinity binding sites for the analogs and that the hydrolysis of TNP-ATP occurs at about the same rate at each of the two sites.

EXPERIMENTAL PROCEDURES

Materials

TNP-ATP, TNP-ADP, and TNP-AMP were obtained from Molecular Probes, Plano, Texas, or synthesized (TNP-ATP) as described (9). TNP-adenine nucleotides were purified as described below. NBDCI was synthesized as described (11). Pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, and adenylate kinase were purchased from Boehringer. Lactic dehydrogenase, Coomassie blue G-250 (brilliant blue G), CDTA (trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid), MES, and NAD⁺ were obtained from Sigma. ADP was obtained from Worthington, crystallized bovine albumin from Pentex, and hexokinase from Miles. EEDQ and DCCD were purchased from Aldrich. Carrier-free \(^{32}P\) (enzyme grade) was obtained from ICN Isotope and Nuclear Division. \(^{32}P\) was a product of New England Nuclear. Thin layer chromatography plates coated with cellulose (Avicel, 100 μ) were purchased from Analtech, and plates coated with polyethyleneimine cellulose (polygram Cel 300 FEI) were obtained from Brinkmann. One-mL plastic tuberculin syringes (Plastipak 5002) were obtained from Becton Dickinson Co. Charcoal (activated carbon, grade S-51) was purchased from Darco Dept., Atlas Powder Co. Finnpipettes were obtained from Variable Volumetrics, Wilmington, MA. Efrapeptin was a gift from Dr. R. L. Hamill, Lilly Research Laboratories, Indianapolis, IN.

Methods

ETP(\(Mg^{2+}\)) (12) and F1 (13) were prepared as described. Before use, F1 was equilibrated with selected buffers and freed of exchangeable nucleotides using the centrifuge column method (14). Two differences in the nature of the bound nucleotide were detected: ethoxy carbonyl-2-ethoxy-1,2-dihydroquinoline; DCCD, N,N'-dicyclohexylurea; ETP(\(Mg^{2+}\)), submitochondrial particles prepared in the presence of \(Mg^{2+}\); AMP-P(NH)P, adenylyl-3'-yl imidodiphosphate.
ferent buffers were employed in these experiments. CDTA buffer contained 0.25 M sucrose, 40 mM MES, 40 mM Tris, 2.5 mM CDTA, and 1 mM KH₂PO₄. The pH was adjusted to 7.5 with Mg buffer, the same as CDTA buffer except that 10 mM MgSO₄ replaced CDTA.

**Assays**

The assay medium for the measurement of kinetics of ATP hydrolysis contained, in a final volume of 1.0 ml, 50 mM Tris-SO₄, pH 8.5, 5 mM phosphoenolpyruvate, 32 μg of pyruvate kinase, 1.7 μg of F₁, 1 mM MgSO₄, and 10 nmoles of MgATP which spanned the range 0.15 to 2.0 mM. MgATP was added in the form of an equimolar complex of MgSO₄ and ATP. The reaction mixture for measurement of the inhibition of ATPase activity by TNP-ATP and TNP-ADP was identical except that 3.5 to 28 nM nucleotide analog also was present. F₁ was added last to a reaction mixture at 30 °C, incubation was continued for 10 min, and the reaction was stopped by adding sulfuryl molybdate (15). P₃ formed was determined colorimetrically (16).

The kinetics of hydrolysis of ATP by ETPh(Mg⁺) was measured in the same reaction mixtures used for F₁, except for the addition of 185 μg of ETPh(Mg⁺) and, when added, TNP-ATP in the concentration range 7.1 to 56.8 nM. The reaction was stopped with 0.1 ml of 50% trichloroacetic acid, precipitated protein was removed by centrifugation, and P₃ formed was determined colorimetrically (16).

ATP synthesis during oxidative phosphorylation catalyzed by ETPh(Mg⁺) was performed as described (17) with ADP in the concentration range 19 to 94 μM and when added, TNP-ADP at 0.8 and 1.6 μM. The ATP-dependent reduction of NAD⁺ by succinate (18) and the exchange between β³P and ATP (18) catalyzed by ETPh(Mg⁺) were measured as described.

The measurement of hydrolysis of TNP-³²PATP and isotope trapping experiments were carried out at room temperature in flat bottom glass tubes (1.5 cm diameter x 4.5 cm high) with vigorous magnetic stirring. The reaction was started by rapidly injecting 100-μl portions of F₁ and nucleotides from a 100-μl Hamilton syringe into 0.9 ml of rapidly stirred buffer which contained Mg⁺⁺ and other compounds as indicated in the legends to the tables and figures. The possibility of mixing artifacts in isotope trapping experiments (19) was minimized by using conditions similar to those described by Ray and Long (20). The latter authors have shown that very fast mixing times (0.2 ms) could be achieved with succinate injection of an enzyme substrate complex into a quenching solution if only a small dilution of the injected solution was required. After each use, the syringe was rinsed with 25 mM CDTA, pH 7.5. The reaction was stopped by adding 100 μl of 60% perchloric acid from a 50-250 μl Finnpipette. The quenched reaction mixtures were stored in an ice bath until the next addition (5-15 min). The samples were transferred to conical centrifuge tubes containing 0.4 ml of a suspension of acid-washed charcoal (250 mg/ml) in 0.1 N HCl. After brief stirring on a Vortex mixer, the samples were centrifuged for 5 min in an IEC clinical centrifuge. The ³²P content of a 0.3-ml sample of the supernatant was determined by liquid scintillation counting (14). Using these extraction conditions, TNP-adenine nucleotides bound by F₁ remained in the supernatant.

The protein content of F₁ solutions was determined by a Coomassie blue G-250 dye-binding procedure (21) using crystalline bovine serum albumin as standard. The protein content of ETPh(Mg⁺⁺) preparations was determined by a modified biuret procedure (16).

The molecular weight of F₁ was taken to be 347,000 in all calculations (22). A unit of ATPase or TNP-ATPase activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol of ATP or 1 μmol of TNP-ATP per min under the described conditions.

**Fluorescence Titrations**

The increase in fluorescence which occurred when TNP-adenine nucleotides were bound by F₁ was measured in a spectrofluorometer with dual monochromators (23). For quantitation of ligand binding to F₁, the excitation wavelength was 374 nm. In this case the emission monochromator was removed, and the emitted fluorescent light was passed through a Corning CS-3-71 filter before reaching the multiplier phototube. At the beginning of the titration, 2.5 ml of a solution containing 0.2 μM F₁ in CDTA buffer or Mg buffer was transferred to a cuvette (1 X 1 cm cross section). TNP-adenine nucleotides were added in increments of up to 0.5 μl from disposable glass micropipettes. Rapid stirring was achieved with the aid of a magnetic stirring bar positioned on the side wall of the cuvettes (24). The recorder trace, representing increased fluorescence, was allowed to reach a stable plateau value after each addition.

To relate observed changes in fluorescence intensity to nanomoles of TNP-adenine nucleotide bound by F₁, 1 nmol of the analog in 2.5 ml of CDTA or Mg buffer, as indicated, was titrated with multiple additions of 0.2 nmol of F₁ in the same buffer. The fluorescence intensity observed in the plateau region where additional F₁ failed to cause further increase in fluorescence was taken as the fluorescence increment. The fluorescence observed when 1 nmol of TNP-adenine nucleotide was completely bound by F₁. Fluorescence data were corrected, when appropriate, for the small fluorescence contributed by F₁ for volume changes during titrations, and for inner filter effects (25).

**TNP-Adenine Nucleotide Binding by F₁**

The reaction mixture contained in a final volume of 0.5 ml, 0.2 μM F₁, ³²P-labeled TNP-adenine nucleotides as indicated, and CDTA or Mg⁺⁺ buffer. The binding reaction was carried out at room temperature (25 °C) for 20 min in order to ensure that binding equilibrium had been reached. The amount of radioactive ligand bound to protein was determined on 150-μl samples using the centrifuge column procedure (14). The Sephadex in the column was equilibrated with the same buffer used in the binding reaction. Radioactivity was measured by liquid scintillation counting (14) using a Beckman LS55 counter. When necessary, windows were optimized for dual label (³²P and β³P) counting. Separate portions of the reaction mixture were passed through identical centrifuge columns for measurement of the recovery of protein. Recovery of F₁ from columns equilibrated with CDTA buffer was approximately 80%. An additional sample of the reaction mixture (100-150 μl) also was taken to determine the initial concentration of radioactive ligands.

**Preparation and Purification of ³²P-labeled TNP-Adenine Nucleotides**

TNP-[γ-³²P]ATP, TNP-[β-³²P]ADP, and TNP-[β-³²P]ATP were synthesized using a variation of the exchange procedure of Gunton and Chappell (26). For TNP-[γ-³²P]ATP synthesis the reaction mixture contained, in a final volume of 1 ml, 100 mM Tris-Cl, pH 8.0, 6 mM MgSO₄, 1 mM dihydrotheo, 1-3 mM chromatographically pure TNP-ATP, 1 mM 3-phosphoglyceric acid, 0.1 mg of phosphoglycerate kinase, and 0.1 mg of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (added in the order given). To this mixture was added 8 x 10⁶ cpm of carrier-free (³²P)phosphoric acid. After 2-4 h at room temperature, the TNP-[γ-³²P]ATP was purified by the following procedure. (This method was also used to purify 1 to 5 μmol quantities of nonradioactive TNP-adenine nucleotides).

Step 1—The reaction mixture (or samples containing TNP-adenine nucleotides in water) was applied to a 4-cm high column of water-washed DEAE-cellulose in a 1-ml plastic tuberculin syringe fitted with a porous polyethylene disk. All TNP-adenine nucleotides remained at the top of the column.

Step 2—TNP-AMP, β³P, and other components of the reaction mixture were eluted from the column by washing with 5 to 10 ml of 1 M formic acid. The progress of the elution was monitored with a survey meter (³²P) and by observing the migration of the yellow band of TNP-AMP.

Step 3—The column was next washed with 10 ml of 3 M formic acid during which TNP-ADP migrated as a broad yellow band. All TNP-adenine nucleotides remained at the top of the column.

Step 4—TNP-ATP was eluted next by washing the column with 10 ml of 2 M ammonium formate, pH 8.2. TNP-ATP moved as an intensely orange band.

Step 5—The eluate from step 4 (2-3 ml) was applied to a 4-cm column of Sephadex LH-20 packed in H₂O in a 1-ml plastic tuberculin syringe. During sample application the column shrank to 2.5 cm, and a narrow (0.5- to 1-cm) band at the top of the column. The column was then washed with 0.5 ml of 100 mM Tris-Cl, pH 8.0. TNP-ATP was subsequently eluted by washing the column with 5 ml of a solution of 50% ethanol in H₂O. The purified compounds were evaporated to dryness and stored at -20°C.

TNP-[γ-³²P]ATP purified by this procedure, as well as nonradioactive TNP-ADP from step 3, migrated as single orange components when chromatographed on polyethyleneimine cellulose plates as described below.
TNP-β-32P]adenine nucleotides were synthesized by a procedure involving adenylic kinase-catalyzed β-γ positional scrambling of the 32P label in TNP-γ-32P]ATP, followed by F, cleavage at the γ phosphorus to yield TNP-β-32P]ADP. The latter was phosphorylated when desired to yield TNP-β-32P]ATP. The reaction mixture for the synthesis of the starting TNP-γ-32P]ATP was the same as described above. At the end of the 4-h incubation, 0.1 mg of adenylic kinase and 1 mM MgSO4 were added, and the mixture was incubated for an additional 60 min. TNP-β-32P]ADP and TNP-β-γ-32P]ATP were then isolated from the reaction mixture using the complete procedure described above with the omission of step 3. The eluate (which contained TNP-β-32P]ADP and TNP-β-γ-32P]ATP) was dissolved in 1 ml of 50 mM Tris-acetate, pH 8.0, containing 10 mM MgSO4. To this was added 1.2 mg of F, in a buffer which contained 0.25 M sucrose and 50 mM Tris-acetate, pH 8.0. After about 60 min, all of the TNP-ATP present was hydrolyzed to TNP-ADP, as determined by chromatography on polyethyleneimine cellulose plates. The mixture was then heated in a boiling water bath for 10 min to precipitate F, which was removed by centrifugation. TNP-β-32P]ADP was purified using steps 1-3 above. The pH of the eluate from step 3 was brought to 8 to 9 with NH4OH before application to the column in step 5.

When TNP-β-32P]ATP was the desired final product, TNP-β-32P]ADP was phosphorylated with phosphoglycerate kinase. In this case, TNP-β-32P]ADP obtained immediately after heating was avoided by incubating F, above was incubated with 10 mM ATP, 1 mM phosphoglyceric acid, and 0.1 mg of phosphoglycerate kinase. After 60 min of incubation at room temperature, TNP-β-32P]ATP was isolated by steps 1 through 5 above. 32P-Labeled TNP-adenine nucleotides were synthesized by the same procedures as described above.

Quantitative association of the 32P label with either the β or the γ position of radioactive TNP-ATP was verified by treating the compound with F, followed by resolution of the products by chromatography on polyethyleneimine cellulose plates. The reaction mixture in a final volume of 100 µl of Mg buffer contained 100 µM 32P-labeled TNP-ATP and 50 µg of F,. After 30 min of incubation at room temperature, 5 µl of sample was applied to the plate and chromatographed as described below.

Analytical thin layer chromatography of TNP-adenine nucleotides was carried out on polyethyleneimine cellulose plates developed for 60 min with a solvent containing 2 M formic acid and 0.5 M LiCl. After drying, the chromatograms were exposed to fumes of NH4OH to intensify the orange color to TNP-adenine nucleotide spots. Under ultraviolet light, the deeply orange TNP-adenine nucleotides were readily distinguished from adenine nucleotide (deep blue) and picric acid (black). The RF values in this system were TNP-ATP, 0.02; TNP-AMP, 0.12; TNP-ADP, 0.46; picric acid, 0.16. Picric acid is readily distinguished from adenine nucleotide (deep blue) and picric acid (black). The RF values in this system were TNP-ATP, 0.02; TNP-AMP, 0.12; TNP-ADP, 0.46; picric acid, 0.16. Picric acid is yellow under room light.

Stock solutions of TNP-adenine nucleotides were prepared by dissolving the dry material in water. The concentration of samples dissolved in 100 mM Tris-Cl, pH 8.0, was determined from the molar absorption coefficients, ε240 = 20,400 M-1 cm-1 and ε259 = 18,500 M-1 cm-1 (9).

RESULTS

Inhibitory Properties of Trinitrophenyl Adenine Nucleotides—TNP-ATP and TNP-ADP inhibited all of the F1-catalyzed reactions tested (Table I). TNP-ATP was a potent competitive inhibitor of the hydrolysis of ATP catalyzed by F,. A slope replot, which was linear, showed a K, of 5.5 nM. A Henderson-type plot (28), designed to correct for inhibitor depletion with tightly binding inhibitors, gave a slightly altered K, of 4.5 nM. Thus inhibitor depletion was not a significant problem in our determination of K,. The TNP-ATP-inhibited rate of ATP hydrolysis was linear, in contrast to the results obtained with the ATP analog AMP-P(NH)P, which required 2-3 min to reach full inhibition of ATP hydrolysis (18). TNP-ADP inhibited competitively the ATPase reaction catalyzed by F,. The inhibited rate of hydrolysis was linear. In contrast to data with TNP-ATP, the slope replot used to calculate K, was concave-up. A K, of <10 nM was determined from the linear portion of the replot. The ATPase reaction catalyzed by ETPH(Mg2+) was also competitively inhibited by TNP-ATP, K, = 21 nM.

TNP-AMP was at least 10,000-fold less potent as an inhibitor than was TNP-ADP. TNP-AMP (26 µM) caused a 16% inhibition of the ATPase reaction catalyzed by F, when 500 µM MgATP was the substrate. In contrast, 2.8 nM TNP-ATP caused 50% inhibition at the same substrate concentration. Trinitrophenol (100 µM) slightly increased the rate of ATP hydrolysis by F, (20-40%), an effect previously observed with 2,4-dinitrophenol (16).

TNP-ADP inhibited ATP synthesis during oxidative phosphorylation catalyzed by ETPH(Mg2+). The inhibition was competitive versus ADP, K, = 1300 nM. TNP-ADP was also a weak inhibitor of the ATP-dependent reduction of NAD" by succinate catalyzed by ETPH(Mg2+). TNP-ADP (320 nM) caused a 50% inhibition of this reaction when 40 µM ATP was used as substrate. A K, was not determined. TNP-ADP (4.1 µM) inhibited a 60% inhibition of the "P-ATP exchange reaction catalyzed by ETPH(Mg2+) when the substrates were 2 mM ATP, 50 µM ADP, and 20 mM 32P, A K, was not determined.

Physical Interactions of TNP-Adenine Nucleotides with F,—The absorbance spectrum of TNP-ATP was altered on mixing with F, in CDTA buffer (Fig. 1A). The broad absorbance peak at 470 nm was reduced in intensity, the peak at 408 nm was reduced in intensity and shifted to 420 nm, and the peak at 259 nm was also reduced. We observed a similarly
altered absorbance spectrum when TNP-ATP and F₁ were mixed in buffer containing MgSO₄ rather than CDTA (not shown), except that the 408-nm peak was shifted to 418 nm. In both buffers the alterations in the absorbance spectra generated difference spectra that could be used to follow binding of nucleotide to protein. Fig. 1B shows difference spectra obtained when 2 and 4 μM TNP-ATP were mixed with 2 μM F₁ in Mg buffer. Both of the spectra show a trough at 480 nm, a peak at 392 nm, and a trough at 394 nm. The corresponding difference spectra in CDTA buffer were similar, except that the trough at 480 nm was smaller, the peak was located at 425 nm and was less intense, and the second trough centered on 394 nm and was deeper. The difference spectra generated by TNP-ATP binding to myosin (9) were very different from those observed here.

We used difference absorbance spectra to determine the number of TNP-ATP binding sites on F₁ in CDTA buffer. The parameter measured in these titrations was the difference in absorbance between the peak at 425 nm and the trough at 394 nm. At concentrations of TNP-ATP below that of F₁, the titration curve was linear (Fig. 2A), suggesting that all of the added TNP-ATP was bound to F₁ (this point is confirmed elsewhere). At concentrations of TNP-ATP greater than that of F₁, a second binding site was filled with lower affinity. A plateau value was reached at 7 μM TNP-ATP. An extrapolation of the linear portion of the titration curve (at low TNP-ATP concentrations) intersected an extrapolation of the plateau value (the equivalence point) at a TNP-ATP:F₁ mol ratio of 2. Thus there are 4 sites on F₁ for TNP-ATP.

The weak fluorescence of TNP-ATP in aqueous solution (10) was enhanced approximately 7-fold by binding to F₁. In addition, the fluorescence maximum of TNP-ADP in Mg buffer was shifted from 555 to 550 nm when equimolar F₁ was added. These observations may be compared with the 10- to 15-fold fluorescence enhancement and the shift in emission maximum from 560 to 548 nm that occurred when TNP-adenine nucleotides were bound by (Na⁺+K⁺)-ATPase (10).

The rate at which the fluorescence increased when TNP-adenine nucleotides were mixed with F₁ depended both on the analog and on the buffer used. A biphasic increase in fluorescence occurred when TNP-ATP interacted with F₁ in CDTA or Mg buffer. The first phase, which was rapid (0.6 s or less), represented interaction of 1 eq of analog with the enzyme. The second phase, which required 2 to 5 min for completion, represented interaction of a second equivalent. A similar biphasic response occurred when TNP-ATP and F₁ were mixed in CDTA buffer. However, when TNP-ATP and F₁ were mixed in Mg buffer, a fluorescence increase representing the interaction of 2 eq of analog with protein was complete within the mixing time of the apparatus (0.6 s).

Binding of TNP-adenine nucleotide to the first binding site on F₁ (Site 1) was complete under the conditions used. A calculation of Kₛ was thus not possible. The remainder of the titration curve was analyzed by Scatchard-type plots (29). The data show that 1.9 sites on F₁ bound TNP-ATP in CDTA buffer with a Kₛ for the looser site (Site 2) of 20 nM (data for Site 1 are not shown). Similarly, a titration with TNP-ADP in Mg buffer also demonstrated 1.9 binding sites for the nucleotide on F₁ (Fig. 2B). The Kₛ for Site 2 was 20 nM.

Direct measurement of the binding of β- or γ-32P-labeled TNP-adenine nucleotide to F₁, was carried out using the centrifuge column procedure (14). All of the added TNP-[γ-32P]ATP bound to F₁ in CDTA buffer when F₁ was in molar

**Fig. 2. Binding of TNP-adenine nucleotides to F₁.** A, difference spectrum titration of TNP-ATP binding to F₁. Difference spectra between 300 and 600 nm at each concentration of TNP-ATP were taken in a Cary 219 spectrophotometer as described in Fig. 1. The reference cell contained the indicated concentration of TNP-ATP in 1.0 ml of CDTA buffer. The sample cell contained the indicated concentration of TNP-ATP and 2 μM F₁ in 1.0 ml of CDTA buffer. The additions of TNP-ATP were made by glass micropipette, and 3-5 min were allowed for the absorbance to stabilize after each addition. The differences (in absorbance units) between the peak in the difference spectrum at 425 nm and the trough at 394 nm is plotted versus the concentration of TNP-ATP added. B, fluorescence titrations of TNP-adenine nucleotide binding to F₁. The reaction mixture for TNP-ATP binding (×—×) contained 0.50 μmol of F₁ in 2.5 ml of CDTA buffer. The reaction mixture for TNP-ADP binding (●—●) contained 0.46 μmol of F₁ in 2.5 ml of Mg buffer. TNP-adenine nucleotides were added in increments of 3 μl, and the resulting fluorescence was measured as described under "Experimental Procedures." The data were used to construct a Scatchard-type plot (29). ½ mol of bound TNP-adenine nucleotide per μmol of F₁, n(μ), mol of bound TNP-adenine nucleotide per μmol of F₁ divided by the concentration of free TNP-adenine nucleotide. C, Scatchard-type plots (29) of 32P-labeled TNP-adenine nucleotide binding measured by the centrifuge column procedure. The reaction mixture for TNP-[γ-32P]ATP binding (curve 1, ×—×) contained 0.105 μmol of F₁ in 0.5 ml of CDTA buffer. The reaction mixture for TNP-[β-32P]ADP binding (curve 2, ●—●) contained 0.105 μmol of F₁ in 0.5 ml of Mg buffer. Twenty min was allowed for binding equilibrium, followed by separation of free and bound ligands by the centrifuge column method as described under "Experimental Procedures." Columns were equilibrated with CDTA buffer for TNP-ATP binding and with Mg buffer for TNP-ADP binding.
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excess. A $K_d$ for this first site could not be calculated. Binding equilibrium was achieved in less than 30 s. At concentrations of TNP-[γ-32P]ATP in excess of F1, about 20 min was required to reach binding equilibrium. A Scatchard-type plot (29) of TNP-[γ-32P]ATP binding to F1, in CDTA buffer is shown in Fig. 2C. 1.5 sites on the enzyme bound the nucleotide. The $K_a$ for Site 2 was 70 nM. A binding titration with TNP-[γ-32P]-ADP demonstrated 1.8 binding sites on F1, with a $K_a$ of 80 nM for Site 2 (Fig. 2C).

We also used the centrifuge column procedure to determine the rate at which enzyme-bound TNP-[β-32P]ATP equilibrated with nonradioactive TNP-ATP in the medium. F1 was first allowed to reach binding equilibrium with amounts of TNP-[β-32P]ATP sufficient to occupy either 0.5 or 1.6 sites on the enzyme. Upon subsequent addition of excess nonradioactive TNP-ATP, TNP-[β-32P]ATP was lost to the medium. F1 was allowed to reach binding equilibrium with amounts of TNP-[γ-32P]ATP in excess of F1, about 20 min was required for binding equilibrium. A Scatchard-type plot (29) of TNP-[γ-32P]ATP in excess of F1, about 20 min was required for Site 1 to reach binding equilibrium. A Scatchard-type plot (29) of TNP-[γ-32P]ATP in excess of F1, about 20 min was required for this first site could not be calculated. Binding equilibrium was achieved in less than 30 s. At concentrations of TNP-[γ-32P]ATP in excess of F1, about 20 min was required to reach binding equilibrium. A Scatchard-type plot (29) of TNP-[γ-32P]ATP binding to F1, in CDTA buffer is shown in Fig. 2C. 1.5 sites on the enzyme bound the nucleotide. The $K_a$ for Site 2 was 70 nM. A binding titration with TNP-[γ-32P]-ADP demonstrated 1.8 binding sites on F1, with a $K_a$ of 80 nM for Site 2 (Fig. 2C).

Properties of the Two Hydrolytic Sites on F1—When F1 was mixed with TNP-[γ-32P]ATP in Mg buffer, the β-γ phosphate bond was hydrolyzed. Chromatography of the reaction products on polyethyleneimine cellulose plates showed components which migrated as $^{32}$P, and TNP-ADP. Kinetics experiments (Fig. 4) determined that TNP-[γ-32P]ATP was hydrolyzed at a maximal velocity of 1.1 mol of $^{32}$P, formed/mol of F1/s, 0.19 unit/mg of protein. The Lineweaver-Burk plot of the data was linear, and a $K_m$ of 0.2 μM was determined. This preparation of F1 hydrolyzed ATP at a maximal velocity of 700 mol of P1, formed/mol of F1/s, 120 units/mg of protein. The $K_m$ for ATP measured in the same experiments was 0.2 μM (not shown). Thus although F1 showed a 1000-fold lower $K_m$ for TNP-ATP as compared to ATP, the modified nucleotide was hydrolyzed 640 times more slowly than ATP.

To ensure that TNP-ATP hydrolysis was catalyzed by F1, and not by a contaminating enzyme, we tested the inhibitor sensitivity of the reaction. All of the compounds or conditions which inhibited the ATPase activity of F1 inhibited the TNP-ATP phosphohydrolase activity to a similar extent (Table II). Treatment of F1 with the covalent modifiers NBD-Cl (30), DCCD (31), or EEDQ (32) inhibited both the ATPase reaction and the hydrolysis of TNP-[γ-32P]ATP. Incubation of F1 solutions in an ice bath for 45 min also resulted in a decrease in both activities. The peptide inhibitor efrapeptin (33), which inhibited the ATPase reaction by 83%, was 100% effective when TNP-[γ-32P]ATP was the substrate. As shown in Table II, ATP was a weak inhibitor of TNP-[γ-32P]ATP hydrolysis. F1 hydrolyzed TNP-[γ-32P]ATP slowly when no divalent metal cation was added and the chelator CDTA was present. Under these conditions, hydrolysis of TNP-[γ-32P]ATP proceeded at a rate of approximately 0.01 mol of $^{32}$P, formed/mol of F1/min (not shown). In contrast, the rate of hydrolysis in Mg buffer was 66 mol of $^{32}$P formed/mol of F1/min.

The technique of isotope trapping was used (19) to determine the minimum number of hydrolytic sites on F1. The experiment is based on observations presented in this paper that F1 can bind 2 molecules of TNP-[γ-32P]ATP in CDTA buffer, that the bound nucleotides equilibrate relatively slowly with medium nucleotides, and that hydrolysis of TNP-[γ-32P]ATP by F1 is very slow in CDTA buffer containing no

![Fig. 3. Equilibration of enzyme-bound TNP-[β-32P]ATP with free TNP-ATP](image)

![Fig. 4. Kinetics of hydrolysis of TNP-[γ-32P]ATP by F1](image)
Inhibition of hydrolysis of ATP and TNP-[γ-32P]ATP

ATPase activity was measured in a 10-min reaction as described (16). The reaction mixture for TNP-[γ-32P]ATP hydrolysis contained 2.5 μmol of TNP-[γ-32P]ATP in 0.95 ml of Mg buffer (except as noted below). Fifty-μl portions of F1 solutions as noted were injected into the reaction mixture, and hydrolysis was allowed to proceed for 10 s. The reaction was stopped by adding 100 μl of 50% perchloric acid, and 32P, released was analyzed as described under "Experimental Procedures."

The reaction mixtures for the covalent modification of F1, contained, in a final volume of 0.5 ml, 0.12 mg of F1, 0.25 mM sucrose, 50 mM Tris-acetate, pH 7.5, 2 mM EDTA, and 40 mM ATP. Modification was carried out by adding 5 μl of an ethanolic solution of NBD-Cl, EEDQ, or DCCD to yield a 0.2 mM final concentration of the modifier used. The mixtures were then incubated for 30 min at 30°C. Ten-μl portions were then used to assay ATPase activity, and 50-μl portions were used to measure TNP-[γ-32P]ATP hydrolysis. Rates for a similarly treated but unmodified F1 solution were ATPase (137 units/mg of F1) and TNP-ATPase (0.9 mol hydrolyzed/mol of F1-s). These values represent 100% activity.

The reaction mixture for the cold treatment of F1, contained, 0.12 mg of F1, and 2 mM ATP in 0.5 ml of CDTA buffer. The mixture was incubated for 45 min in an ice water bath. Ten-μl portions were assayed for ATPase activity, and 50-μl portions were used to measure hydrolysis of TNP-[γ-32P]ATP as described above. The rates for a duplicate F1 solution kept for 45 min at 25°C were ATPase, 120 units/mg of F1, and TNP-ATPase, 0.9 mol hydrolyzed/mol of F1-s. These values represent 100% activity.

Reaction medium for efrapeptin inhibition contained 1.5 μg of efrapeptin, 0.12 mg of F1, and 4 mM ATP in a 0.5-ml final volume of CDTA buffer. Ten-μl portions were assayed immediately for ATPase activity, and 50-μl portions were used to measure TNP-[γ-32P]ATP hydrolysis as described above. Rates for a duplicate F1 solution without efrapeptin were ATPase, 120 units/mg of F1, and TNP-ATPase, 0.9 mol hydrolyzed/mol of F1-s.

To measure the effect of 5 mM ATP on TNP-[γ-32P]ATP hydrolysis, 10-μl amounts of CDTA buffer containing 6.7 pmol of F1 were injected into 0.99 ml of rapidly stirred Mg buffer containing 5 mM ATP and 0.2 nmol of TNP-[γ-32P]ATP. The reaction rate was calculated from a series of points taken at 2-s intervals for 10 s. The rate in the absence of ATP was 0.7 mol of 32P, released/mol of F1-s. With ATP the rate was the 0.29 mol of 32P released/mol of F1-s.

### Table II

**Table II**

<table>
<thead>
<tr>
<th>Addition or treatment</th>
<th>Rate of hydrolysis</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>TNP-[γ-32P]ATP</td>
<td></td>
</tr>
<tr>
<td>0.2 mM NBD-Cl, 45 min</td>
<td>2.0</td>
<td>5</td>
</tr>
<tr>
<td>0.2 mM EEDQ, 45 min</td>
<td>29.0</td>
<td>48</td>
</tr>
<tr>
<td>0.2 mM DCCD, 45 min</td>
<td>20.0</td>
<td>37</td>
</tr>
<tr>
<td>0°C cold treatment, 45 min</td>
<td>68.0</td>
<td>74</td>
</tr>
<tr>
<td>Efrapeptin, 17 pg</td>
<td>17.0</td>
<td>0</td>
</tr>
<tr>
<td>5 mM ATP (in assay)</td>
<td>17.0</td>
<td>41</td>
</tr>
</tbody>
</table>

The reaction mixtures for the covalent modification of F1 contained, 0.12 mg of F1, and 4 mM ATP in a 0.5-ml final volume of CDTA buffer. Ten-μl portions were assayed immediately for ATPase activity, and 50-μl portions were used to measure TNP-[γ-32P]ATP hydrolysis as described above. Rates for a duplicate F1 solution without efrapeptin were ATPase, 120 units/mg of F1, and TNP-ATPase, 0.9 mol hydrolyzed/mol of F1-s. These values represent 100% activity.

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### FIG. 5

**FIG. 5. Isotope trapping titration of catalytic sites on F1.**

The reaction mixtures contained, in a final volume of 0.5 ml of CDTA buffer, 0.5 nmol of F1 and the indicated concentration of TNP-[γ-32P]ATP. A separate mixture was prepared for each concentration of radioactive analog. After 20 min of incubation, samples of 100 μl were removed for measurement of analog binding by the centrifuge column method. In both experiments F1 was found to be essentially free of 32P. The isotope trapping experiments thus identify a species of F1 in which the 2 available TNP-ATP binding sites are occupied. This enzyme, which retains its activity throughout the experiment, hydrolyzes the bound nucleotide at rates comparable to the rate at high concentrations resulted in at least 1.5 mol of TNP-[γ-32P]ATP bound/mol of F1. The binding curve was closely followed by the curve for hydrolysis of bound TNP-[γ-32P]ATP in the isotope trapping experiment, which showed 1.4-1.7 mol of TNP-[γ-32P]ATP hydrolyzed/mol of F1. The close correspondence between the two curves provides evidence that previously bound TNP-[γ-32P]ATP was hydrolyzed without dissociating from the enzyme.
which it hydrolyzes free TNP-ATP (1.1 mol of P, formed/mol of F1, s) and discharges both products. We conclude that F1 has at least 2 catalytic sites for TNP-ATP hydrolysis.

The initial rate of hydrolysis at each of the 2 sites was measured separately using the isotope trapping method. Fig. 6 (crosses) shows the rate of hydrolysis at Site 1. Site 1 was filled by incubating 20 fl of F1, 1.5 mM nonradioactive TNP-ATP, and 0.5 mM Mg buffer containing in addition, 30 fM effrapeptin. After 5 min of incubation, 22.5 nmol of TNP- [γ-32P]ATP was added to each mixture to start the binding reaction. At the indicated times, 100-μl samples were removed to measure binding by the centrifuge column method. The columns were equilibrated with CDTA buffer. Two incubation mixtures were prepared containing, in 1 ml of CDTA buffer, 2.05 μM F1 and 25 μM TNP- [γ-32P]ATP. After 20 min of incubation, 30 nmol of effrapeptin was added to mixture 2 (■—■). An equivalent volume of CDTA buffer (50 μl) was added to mixture 1 (○—○). After 5 min of incubation, 1.5 μM of nonradioactive TNP-ATP was added to each mixture. At the indicated times 100-μl samples were removed for measurement of binding by the centrifuge column method. Binding of TNP- [γ-32P]ATP by F1, is expressed as the percent of the molar binding ratio immediately before the addition of nonradioactive TNP-ATP. In the absence of effrapeptin, 100% binding was equivalent to 1.59 mol of TNP-[γ-32P]ATP/mol of F1. In the presence of effrapeptin, the value was 1.78.

FIG. 7. Efrapeptin inhibition of TNP- [γ-32P]ATP binding and exchange. A. Binding. Two incubation mixtures each containing 1 ml of CDTA buffer were prepared as follows. Mixture 1 (■—■), contained 2.05 μM F1 and mixture 2 (○—○) contained, in addition, 30 μM effrapeptin. After 5 min of incubation, 22.5 nmol of TNP-[γ-32P]ATP was added to each mixture to start the binding reaction. At the indicated times, 100-μl samples were removed to measure binding by the centrifuge column method. The columns were equilibrated with CDTA buffer. Two incubation mixtures were prepared containing, in 1 ml of CDTA buffer, 2.05 μM F1 and 25 μM TNP-[γ-32P]ATP. After 20 min of incubation, 30 nmol of effrapeptin was added to mixture 2 (■—■). An equivalent volume of CDTA buffer (50 μl) was added to mixture 1 (○—○). After 5 min of incubation, 1.5 μM of nonradioactive TNP-ATP was added to each mixture. At the indicated times 100-μl samples were removed for measurement of binding by the centrifuge column method. Binding of TNP- [γ-32P]ATP by F1, is expressed as the percent of the molar binding ratio immediately before the addition of nonradioactive TNP-ATP. In the absence of effrapeptin, 100% binding was equivalent to 1.59 mol of TNP-[γ-32P]ATP/mol of F1. In the presence of effrapeptin, the value was 1.78.

(Efrapeptin Effects on TNP-ATP Binding and Hydrolysis—Efrapeptin, a tight binding peptide inhibitor of F1, (33), affected TNP-ATP binding by the enzyme (Fig. 7). When added to F1, before the nucleotide, efrapeptin inhibited the rate of TNP-[γ-32P]ATP binding (Fig. 7A). When added after nucleotide binding was complete, efrapeptin severely inhibited the rate at which bound TNP-[γ-32P]ATP equilibrated with medium nonradioactive TNP-ATP (Fig. 7B). Efrapeptin also caused an increase in the amount of TNP-[γ-32P]ATP bound as measured by the centrifuge column method.

The inhibitor had a pronounced effect on substrate and product binding in isotope trapping experiments (Fig. 8A). F1 was allowed to bind a mixture of TNP-[γ-32P]ATP and TNP-[β-32P]ADP in CDTA buffer for 20 min, after which time efrapeptin was added. The molar binding ratio was 2.1 mol of labeled TNP-ATP per mol of F1. Mg2+ was then added to start the hydrolysis reaction together with an isotope trap of nonradioactive TNP-ATP. Loss of 32P from TNP-[γ-32P]ATP occurred in a biphasic manner. One equivalent of 32P was lost to the medium within 30 s, and the second equivalent was lost in 30 min (curve 1). During this period the 2P bound to F1 remained constant (curve 2). Thus product TNP-[β-32P]ADP did not dissociate from the enzyme during the experiment.
Separate experiments to measure hydrolysis of bound TNP-[γ-32P]ATP also showed a biphasic curve when efrapeptin was present (Fig. 8B). Curve 1 shows that the rate of 32P appearance in the medium was equal to the rate of loss of TNP-[γ-32P]ATP from the enzyme shown in Fig. 8A (curve 1). 32P (1.9-2.1 mol) was released per mol of F₃. Fig. 8B (inset) is a separate hydrolysis experiment performed on an expanded time scale. The first phase of the reaction is nearly complete within 2 s and is clearly distinguished from the second phase.

We also performed a hydrolysis experiment without an isotope trap. If efrapeptin allowed multiple turnovers of F₃ to occur, this experiment would show hydrolysis of the 14-fold molar excess of TNP-[γ-32P]ATP carried over from the binding step. The result (Fig. 8B, curve 2) showed 2.1-2.5 mol of 32P released/mol of F₃, indicating that F₃ did not hydrolyze appreciable medium TNP-[γ-32P]ATP in the presence of efrapeptin.

Further experiments with efrapeptin revealed which of the 2 catalytic sites was responsible for the rapid hydrolysis of the first equivalent of TNP-[γ-32P]ATP noted in Fig. 8B (inset). Site 1 was partially filled with radioactive analog (0.8 mol/mol of F₃), and Site 2 was filled by incubating first with excess nonradioactive TNP-ATP for 1 min and then with efrapeptin (Fig. 9, curve 1). Addition of the enzyme-substrate-efrapeptin complex to Mg buffer containing an isotope trap was followed by only a slow rate of hydrolysis of TNP-[γ-32P]ATP, which eventually approached completion. This experiment was similar to the one described in Fig. 6 in that some loss of TNP-[γ-32P]ATP from Site 1 occurred during the 1-min incubation to fill Site 2. Immediately after the addition of efrapeptin, the molar binding ratio (TNP-[γ-32P]ATP/F₃) determined in the centrifuge column was 0.53, a loss of about 25% of the added analog. Further loss of labeled nucleotide from Site 1 was, however, dramatically reduced by efrapeptin since 10 min after incubation the molar binding ratio was 0.53.

In a companion experiment, Fig. 9, curve 2, Site 2 was filled with TNP-[γ-32P]ATP, and Site 1 contained the nonradioactive analog. Under the conditions of the experiment, the molar binding ratio of TNP-[γ-32P]ATP/F₃ immediately after adding...
efrapeptin was 0.68. Since approximately 0.25 eq of nonradioactive TNP-ATP would be expected to leave Site 1 during the second incubation (estimated from the experiments of curve 1 and Fig. 3) and the site would probably be reoccupied by TNP-[y-32P]ATP, it is likely that approximately 0.4 eq of presence of efrapeptin rapid hydrolysis of previously bound analog occurs at Site 2.

**DISCUSSION**

The major finding emerging from this study is that soluble, homogeneous beef heart mitochondrial ATPase contains two binding sites, which are hydrolytic sites, for the ATP analog 2',3'-O-(2,4,6-trinitrophenyl)ATP. As shown in Fig. 6, the two sites are about equally effective in catalyzing the hydrolysis of the analog to TNP-ADP and P_i. Although in TNP-ATP hydrolysis by F_i, it is 1000 times lower than that for ATP and the analog is hydrolyzed at a rate 500 times less than the normal substrate, our evidence supports the conclusion that TNP-ATP hydrolysis is catalyzed by F_i itself rather than a contaminant of the enzyme preparation. Thus, exposure of F_i to covalent modifiers, to the peptide efrapeptin, and to cold treatment caused approximately the same percentage of inhibition of the hydrolysis of ATP and of TNP-ATP. In addition TNP-ATP and TNP-ADP were potent competitive inhibitors (versus ADP) of ATP synthesis during oxidative phosphorylation and of ATP hydrolysis catalyzed by F_i and submitochondrial particles. The analogs also inhibited the exchange between 32P and ATP and the ATP-dependent reduction of NAD+ by succinate. These observations, as well as the finding that ATP inhibited the F_i-catalyzed hydrolysis of TNP-ATP, strongly suggest that the TNP-adenine nucleotides interact with the same catalytic sites on F_i which normally interact with ATP. These experiments thus provide the first direct evidence for the presence of two hydrolytic sites on F_i and are consistent with the suggestion that both sites participate in the normal catalytic activity of the enzyme.

The possibility that there might be more than one catalytic site on F_i was earlier suggested by kinetics studies (35), by labeling experiments using a photoaffinity nucleotide (36), by studies on the F_i-catalyzed exchange reaction between H_37P_0 and the oxyns of the y-phosphate group of ATP (4), and by studies of the chloroplast ATPase during photophosphorylation (37). The latter experiments in fact made use of an isotope trap type of approach (38). Further evidence in support of more than one catalytic site is provided by the observations of Senior et al. (8) that only one gene coded for subunit β in the ATPase from E. coli. Since reasonable evidence is available that a hydrolytic site is associated with subunit β (1), the enzyme should have as many hydrolytic sites as β subunits. On this basis, one may expect at least two hydrolytic sites on the enzyme from beef heart mitochondria (1). However, the apparent large difference in binding affinity for TNP-adenine nucleotides between Sites 1 and 2 and the fact that both sites are catalytic, is indicative of negative cooperativity in substrate binding by the enzyme. Thus if a third catalytic site is present on F_i, it might well exhibit an even lower affinity for the analogs. It should be emphasized that while the data presented in this paper are compatible with only 2 hydrolytic sites on F_i, the possibility of additional sites is not ruled out.

Throughout the conduct of the isotope trapping experiments, particular attention was given to the possibility of inadequate trapping or of mixing artifacts (see Ref. 19 for a recent review). In the experiments described in Fig. 5, for example, TNP-[y-32P]ATP was diluted 300- to 60,000-fold in aliquots were added to the trapping mixture containing the nonradioactive analog. Since even a 500-fold isotope dilution would have permitted a maximum of only 0.07 mol of free TNP-[y-32P]ATP to be hydrolyzed by each mol of F_i, it is clear that excellent trapping was achieved. The possibility that a slow approach to complete mixing might have influenced the results (19) also was rendered unlikely through the use of mixing conditions similar to those of Ray and Long (20). Under optimal conditions it would be expected that solutions injected from the syringe into the trapping solution in the experiment of Fig. 5 would have reached 90% of their original concentration in 0.2 ms (20). Mixing times 100-fold longer than this optimal value nevertheless would not have generated an observable mixing artifact in the present system.

The TNP-adenine nucleotide binding properties of the two catalytic sites on F_i are of interest with regard to the mechanism of action of the enzyme. Binding of TNP-ATP or TNP-ADP to Site 1 was stoichiometric with added analog at all concentrations of F_i used in these studies. Consequently, the K_i for Site 1 was not determined. Binding of analog to Site 2 was characterized by a readily measurable K_i. The value calculated was 20 nM in fluorescence titrations (Fig. 2B) and 80 nM in the centrifuge column procedure (Fig. 2C). The lower affinity exhibited by Site 2 in the latter procedure may be caused by weak interactions between TNP-adenine nucleotides and the Sephadex support since the analogs migrated slightly behind ATP during conventional chromatography on columns of Sephadex G-50. TNP-ATP-Sephadex interactions may also have caused the small underestimations of TNP-[y-32P]ATP binding by the centrifuge column procedure (Figs. 2 and 5).

The fluorescence increase observed when F_i was added to excess TNP-ATP in Mg buffer appeared to occur in a single rapid step (less than 0.6 s) and was equivalent in magnitude to the binding of 2 mol of analog per mol of enzyme. It should be pointed out that hydrolysis of TNP-ATP at 2 catalytic sites on F_i, as described in this paper, requires an interaction of substrate with enzyme at least as rapid as that observed in the latter fluorescence experiment. Although only two binding sites were observed in most of the experiments described in this paper, a third site on F_i was filled during prolonged incubation of the enzyme with high concentrations of TNP-adenine nucleotides in Mg buffer. The presence of a third site for AMP-P(NH)_2P and ADP on F_i was observed under similar conditions of incubation. In the presence of CDTA buffer, however, only two binding sites were observed for either TNP-ATP or TNP-ADP. The nature of the third binding site remains undetermined. The possibility that Site 3 is a catalytic site is not ruled out although the slow rate of occupancy is indicative of exchange with one of the tight binding sites on the molecule (39).

In view of the large differences in binding affinity between, for example, ADP (0.3 and 47 μM, see Ref. 1) and TNP-adenine nucleotides, it might be expected that a binding site for the trinitrophenyl group would be adjacent to the nucleotide binding site. However, we did not observe either an experimentally significant difference spectrum or fluorescence enhancement when TNP-AMP was added to F_i. It would appear that TNP-AMP, like AMP (1), does not bind to F_i. In keeping with this suggestion, neither TNP-AMP nor trinitrophenol caused significant inhibition of ATP hydrolysis. Al-

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2 H. S. Penefsky, unpublished observations.
though other interpretations may be invoked, these observations raise the interesting possibility that initial binding of TNP-adenine nucleotides occurs via interactions between the enzyme and the nucleotide moiety followed by a conformational change in the protein that creates a binding site for the trinitrophenyl group.

Hydrolysis of TNP-\([\gamma-^{32}P]\)ATP, whether free in the medium or previously bound to \(F_1\), proceeded at a linear rate and exhibited neither burst nor lag phases. The turnover time for the enzyme was 1.1 s\(^{-1}\). The rate of breakdown of the \(F_1\)-TNP-\([\gamma-^{32}P]\)ATP complexes studied in Fig. 6 approached the theoretical \(t_{1/2}\) of 1.26 s expected for a 2-site enzyme. The \(t_{1/2}\) for either Site 1 or Site 2 alone was the same as the \(t_{1/2}\) for both sites together. It is likely that the rate-limiting step during steady state hydrolysis was breakage of the phosphate ester bond or a step subsequent to binding and prior to bond breakage. If a later step, such as product release, were rate limiting, a burst in the release of \(^{32}\)P should have been observed in the first catalytic cycle.

The identification of at least two hydrolytic sites on the ATPase is immediately relevant to proposed models of enzyme action such as the binding change mechanism (4) and flip-flop mechanisms (6, 7). Direct evidence regarding alternation in the catalytic activity of the two sites is lacking. However, it would appear that a change in the affinity for TNP-adenine nucleotides occurs during the first catalytic cycle since the products of hydrolysis of TNP-ATP in Site 1 are released to the medium when the enzyme is allowed to turn over. In view of the very high affinity of Site 1 for TNP-ATP and TNP-ADP and of the turnover time during hydrolysis of 1.1 s\(^{-1}\), release of the ADP analog must be associated with a rapid and considerable decrease in affinity. It remains to be shown whether alternation in affinity is a recurring feature of catalysis in the steady state.

The possibility of a dynamic interchange in the affinities of Sites 1 and 2 which might allow a TNP-adenine nucleotide originally inserted in the high affinity site (Site 1) to appear as though it was located in the lower affinity site (Site 2) would seem to be ruled out, at least in the absence of Mg\(^{2+}\), by the experiments of Fig. 3. If interchange in affinity occurred, one might expect that the rate of the loss of TNP-ATP bound in Site 1, Fig. 3, curve 1, would be characterized by a biphasic curve, similar in shape to curve 2 of the same figure.

The experiments with efrapeptin are of interest both with regard to the properties of the two catalytic sites and the mechanism of inhibition by the peptide. Addition of efrapeptin to the complex of TNP-ATP and F\(_1\) dramatically reduced the rate at which bound analog exchanged with analog free in the medium (Fig. 8). Efrapeptin also completely prevented the exchange out of \(^{[3}H]ADP\) from one of two binding sites for ADP on F\(_1\). Addition of the peptide to F\(_1\) before ADP permitted only one of the two binding sites for the nucleotide to be filled.\(^2\) These observations suggest that efrapeptin inhibits ATP hydrolysis and oxidative phosphorylation by preventing binding and release of nucleotide at a single catalytic site. It is of further interest that TNP-ATP, bound in Site 2 in the presence of efrapeptin, was hydrolyzed almost completely before any appreciable hydrolysis of analog occurred in Site 1 (Fig. 9). The data indicated that the relationship in affinity between the two catalytic sites was preserved even in the presence of the peptide. Efrapeptin thus permitted a further demonstration of the presence of two catalytic sites on F\(_1\), without the use of an isotope trap.

REFERENCES