Nucleotide Binding by the Epidermal Growth Factor Receptor Protein-tyrosine Kinase

TRINITROPHENYL-ATP AS A SPECTROSCOPIC PROBE*

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The nucleotide binding properties of the epidermal growth factor (EGF) receptor protein-tyrosine kinase were investigated with the fluorescent nucleotide analog 2′(3′)′O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP). TNP-ATP was found to be an active substrate for the autophosphorylation reaction of the recombinant EGF receptor protein-tyrosine kinase domain (TKD). Whereas the V_max for the TNP-ATP-dependent autophosphorylation reaction was approximately 200-fold lower than that of ATP, the K_m for this reaction was similar to that observed with ATP. The nucleotide analog was also shown to be an inhibitor of the ATP-dependent autophosphorylation and substrate phosphorylation reactions of the TKD. Spectroscopic studies demonstrated both a high affinity binding of TNP-ATP to the recombinant TKD and a markedly enhanced fluorescence of the bound nucleotide analog. The fluorescence of enzyme-bound TNP-ATP was attenuated in the presence of ATP, which enabled determination of the dissociation constants for both ATP and the Mn^{2+} complex of ATP. A truncated form of the EGF receptor TKD lacking the C-terminal autophosphorylation domain exhibited an enhanced affinity for TNP-ATP, which indicated that the autophosphorylation domain occupied the peptide substrate binding site of the TKD and modulated the binding of the nucleotide substrates.

A large variety of polypeptide growth factor receptors (1, 2) and oncogene products (3) possess an intrinsic protein-tyrosine kinase activity that is known to be critical to their diverse cellular functions. Each of these enzymes catalyzes the ATP-dependent phosphorylation of tyrosine residues in peptide substrates and most undergo an autophosphorylation reaction, by which specific tyrosine residues within their primary structures become phosphorylated. In some cases, autophosphorylation has been shown to regulate the enzymic activity of protein-tyrosine kinases. In other cases, e.g., for the variety of polypeptide growth factor receptors with protein-tyrosine kinase activity, autophosphorylation is known to trigger the formation of signal transduction complexes (1, 2, 4). Here the phosphorylated protein-tyrosine kinases interact with cellular target proteins that specifically recognize and bind to phosphorylated tyrosine residues (5).

Whereas protein-tyrosine kinases constitute an important family of enzymes with well studied involvement in numerous cell growth control processes, including significantly the aberrant growth of cancerous cells, much remains to be determined about their enzymic mechanisms. Studies of the steady state kinetics of these enzymes have been reported (6–10). However, the mechanisms of the substrate phosphorylation and autophosphorylation reactions of protein-tyrosine kinases are not understood at the molecular level. Also, the structural changes in the active site that accompany the activation of protein-tyrosine kinase activity by various agents have not been characterized.

In the present work, we have investigated the potential of the fluorescent nucleotide analog 2′(3′)′O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) as a molecular probe for enzymological studies of protein-tyrosine kinases. We have determined that TNP-ATP binds to the active site of the epidermal growth factor (EGF) receptor protein-tyrosine kinase, shows a markedly enhanced fluorescence when bound to the active site, and is also a substrate for the autophosphorylation reaction of the protein kinase. Comparison of the affinities of TNP-ATP for full-length and C-terminally truncated recombinant EGF receptor protein-tyrosine kinase domains indicated that the C-terminal autophosphorylation domain may occupy the active site at equilibrium. The fluorescent nucleotide analog was also exploited in the determination of the affinities of binding of both ATP and the Mn^{2+} complex of ATP to the kinase active site. The TNP-ATP nucleotide analog therefore appears to have much potential as a molecular probe for investigating the structure and function of protein-tyrosine kinases.

EXPERIMENTAL PROCEDURES

Materials—2′(3′)′O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was obtained from Molecular Probes, Inc. ATP sodium salt and [Glu:Ala:Tyr]_{3} (GAT), a random copolymer of glutamate, alanine, and tyrosine in the indicated mole ratio and with an average molecular weight 43,000, were purchased from Sigma. (γ^{32-P})ATP (−3,000 Ci/mmol) was supplied by DuPont NEN. RC20, a recombinant antiphosphotyrosine reagent consisting of the phosphotyrosine recognition domain of the PY20 antibody conjugated to the horseradish peroxidase enzyme, was provided by Transduction Laboratories. tyrosine and ATP; EGF, epidermal growth factor; TKD, recombinant EGF receptor protein-tyrosine kinase domain; TKD38, 38-kDa C-terminally truncated TKD; TKD61, 61-kDa full-length TKD; GAT, a 13-amino acid peptide previously characterized as a high affinity substrate for the EGF receptor protein-tyrosine kinase (11), was kindly provided by Dr. Cheryl A. Guyer, Vanderbilt University, Nashville, TN. Expression of EGF Receptor Protein-tyrosine Kinase Domains—The

1 The abbreviations used are: TNP-ATP, 2′(3′)′O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; Mn^{2+}-ATP, complex of Mn^{2+} and ATP; GAT, a random copolymer of glutamate, alanine, and tyrosine; MBP-B3-11, fusion protein comprised of maltose binding protein and an ErbB3 C-terminal peptide; PAGE, polyacrylamide gel electrophoresis; E_p, total enzyme concentration; T_p, total TNP-ATP concentration; K_{NPATP}, dissociation constant for TNP-ATP binding; ΔF, enhancement of fluorescence yield; ΔF_o, difference in intrinsic fluorescence yields of enzyme-bound and free TNP-ATP; S, the peptide substrate tyrsub; K_s, dissociation constant for tyrsub binding.
generation and purification of recombinant human EGF receptor protein-tyrosine kinase domain (TKD) proteins will be described in detail elsewhere. Briefly, two forms of the EGF receptor TKD, each with a Met-His-His-His-His-His-His-His leader peptide, were generated with the baculovirus/insect cell system and purified by metal-chelating chromatography. The TKD61 protein, incorporating amino acid residues 645 to 1186 of the human EGF receptor protein, was expressed in the baculovirus/insect cell system and purified by metal-chelating chromatography. The TKD38 protein incorporated amino acid residues 61 to 453 of the human EGF receptor protein, and exhibited high substrate phosphorylation and autophosphorylation activities.

Comparison of ATP or TNP-ATP as Substrates for the TKD61 Autophosphorylation Reaction—The kinetics of the autophosphorylation reaction of the TKD61 protein were analyzed either by a radiochemical assay ($^{32}$P]ATP as substrate) or by an immunological assay (unlabeled ATP or TNP-ATP as substrate). In the former assay, the TKD61 protein (1.0 µg) was incubated for 10 s at room temperature in 20 mM sodium Hepes, 10 mM MnCl$_2$, 0.1% Triton X-100, pH 7.4, with $(^{32}$P]ATP (10$^{-6}$ cpm/pmol) added to the indicated concentration to initiate the autophosphorylation reaction (final volume 50 µl). The reaction was quenched by addition of 10% trichloroacetic acid, and the incorporation of $^{32}$P into TKD61 was quantified by a filter binding assay as described previously (11).

Alternatively, the autophosphorylation activity of TKD61 with ATP or TNP-ATP as substrate was assayed by an immunological method, employing the recombinant antiphosphotyrosine/horseradish peroxidase conjugate RC20. TKD61 protein (1.0 µg) was preincubated as described above, and the reaction was initiated by addition of either ATP or TNP-ATP to the indicated concentration (final volume 36 µl). After reaction with ATP as substrate, or a 15-min incubation with TNP-ATP as substrate, the reactions were quenched by addition of SDS-PAGE sample buffer, and samples were subjected to SDS-PAGE (12% gel). Resolved proteins were electrochemically transferred to an Immobilon-P membrane (Millipore) and blotted with the RC20 reagent (Amersham). The TKD61 protein, designated TKD61, contained the entire cytosolic domain of the EGFR (amino acids 645 to 1186), and exhibited high substrate phosphorylation and autophosphorylation activities.

Analysis of ATP as an Inhibitor of TKD61 Autophosphorylation and Substrate Phosphorylation Reactions—Aliquots of TKD61 protein (1.0 µg) were assayed for autophosphorylation activity in a 20 mM sodium Hepes, 50 mM sodium chloride, 10 mM MnCl$_2$, 0.1% Triton X-100, pH 7.4 medium, supplemented with varying concentrations of the substrate $(^{32}$P]ATP (0–100 µM) and the inhibitor ATP (0–100 µM) (see above). For each fixed TNP-ATP concentration, the ATP concentration dependence of the autophosphorylation velocity was fit with the Michaelis-Menten equation by nonlinear least squares (Sigma Plot software, Jandel Scientific) to determine the apparent $K_{m}$ and $V_{max}$ for the reaction and best-fit theoretical curve. The velocity data and theoretical curve for each TNP-ATP concentration were then plotted in double-reciprocal form. The inhibition of the substrate phosphorylation activity of TKD61 by ATP was analyzed in a similar manner. Here, aliquots of TKD61 (0.20 µg) were assayed in a 20 mM sodium Hepes, 50 mM sodium chloride, 10 mM MnCl$_2$, 0.1% Tween bovine serum albumin, 10% (v/v) glycerol, pH 7.4 medium, supplemented with 0.4% Tween GAT.

Fluorescence Spectroscopic Analysis of TNP-ATP/EGF Receptor TKD Interactions—Recombinant human EGF receptor TKD proteins and TNP-ATP were diluted to the indicated concentrations in 1.5 ml of 20 mM sodium Hepes, 10% (v/v) glycerol, pH 7.4, in a standard 1-cm fluorescence cell thermostated at 22°C. Fluorescence emission spectra were recorded with an SLM4800C spectrofluorometer (SLM Instruments) in ratio mode. Emission spectra were corrected by subtraction of an appropriate buffer blank.

The affinity of the interaction of TNP-ATP with each of the TKD proteins was determined by titrating a fixed quantity of the TKD protein with increasing concentrations of the fluorescent nucleotide, while recording the fluorescence intensity at the wavelength maximum of the emission spectrum of enzyme-bound TNP-ATP (540 nm) with excitation at 418 nm. A control TNP-ATP titration was performed without addition of the TKD protein, and the obtained fluorescence intensity data were subtracted from that recorded in the presence of TKD protein. The enhancement of fluorescence occurring in the presence of the TKD protein was assumed to be proportional to the quantity of enzyme-bound TNP-ATP and was plotted as a function of total concentration added TNP-ATP (see Fig. 5). The titration curve would then obey the equation

$$\Delta F = 0.5 \cdot \Delta F_0 \cdot \left(1 + \frac{K_{app}}{[\text{TNP-ATP}]} \right)$$

where $\Delta F_0$ and $T_0$ are the total concentrations of added TKD protein and TNP-ATP nucleotide, respectively, $K_{app}$ is the dissociation constant characterizing the interaction, and $\Delta F$ is the difference in the intrinsic fluorescence yields of enzyme-bound and free TNP-ATP. This equation was fit to the titration data with a nonlinear least squares algorithm and with $K_{app}$, $\Delta F_0$ as adjustable parameters. The best-fit parameters were used to generate the theoretical curves shown in Fig. 5. Dissociation constants for binding of the Mn$^{2+}$ complex of TNP-ATP (MnTNP-ATP) were determined by TNP-ATP titrations performed in the presence of a fixed concentration of free Mn$^{2+}$ ion.

Given the known values for $K_{app}$, the dissociation constants for binding of ATP to the TKD proteins ($K_{app}$) were determined by titrating mixtures of TNP-ATP and TKD with increasing concentrations of ATP, while monitoring the fluorescence of bound TNP-ATP (see Fig. 6). Equation 1 was fit to the titration data with $K_{app}$, $K_{0}$, and $K_{app}$ treated as adjustable parameters. Dissociation constants for binding of the Mn$^{2+}$ complex of ATP (MnATP) were determined similarly by ATP titrations in which the concentration of free Mn$^{2+}$ ion was held constant (cf. Ref. 1).

The effect of the peptide substrate tyrsub (S) on the affinity of the nucleotide for the TKD38 protein was analyzed by determining the apparent dissociation constant for TNP-ATP ($K_{TNP-ATP}$) in the presence of varying concentrations of the peptide (see Fig. 7). The peptide appeared not to compete directly with TNP-ATP for binding to the TKD38 protein, but instead reduced the affinity of binding of TNP-ATP. Assuming that S and TNP-ATP bound reversibly to the TKD protein, the dependence of $K_{TNP-ATP}$ on the concentration of S would be given by

$$K_{TNP-ATP} = K_{TNP-ATP} \cdot (1 + [S]/[S]) / z$$

where $K_{TNP-ATP}$ and $K_{0}$ are the dissociation constants for TNP-ATP and S, respectively, and $z$ is the factor by which $K_{TNP-ATP}$ is enhanced in the presence of a saturating concentration of S.

RESULTS

TNP-ATP as an Active Substrate for the EGF Receptor Protein-tyrosine Kinase—The fluorescent nucleotide analog 2'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP) has been shown to bind with high affinity to the active sites of a variety of ATP-dependent enzymes and, in some cases, has been shown to function as an alternate substrate. In the present studies, the potential for TNP-ATP to serve as a substrate for the autophosphorylation reaction of a protein-tyrosine kinase was examined. The cytoplasmic protein-tyrosine kinase domain (TKD) of the EGF receptor, generated with the baculovirus/insect cell system and purified by metal-chelating chromatography, was used in these experiments. This recombinant protein, designated TKD61, contained the entire cytosolic domain of the EGF receptor protein linked by amino acid residues 645 to 1186 and exhibited high substrate phosphorylation and autophosphorylation activities.

The dependence of the autophosphorylation of the TKD61 protein upon the concentration of ATP is shown in Fig. 1, A and B. Similar autophosphorylation kinetics were observed when the reaction was monitored by assaying the incorporation of $^{32}$P into the protein with the substrate $(^{32}$P]ATP (Fig. 1A, $K_{m}$ = 6.0 ± 0.8 µM, n = 3) or by quanti-
MnCl₂ and varying concentrations of ATP were incubated as in the assay. The TKD61 protein was incubated with TNP-ATP (Fig. 1C). The reaction was subsequently assayed by antiphosphotyrosine immunoblotting. The TKD61 protein was shown to be autophosphorylated with a similar nucleotide concentration dependence (Kₘ = 4.4 ± 0.5 μM, n = 6). The maximal velocity for the autophosphorylation of the TKD61 protein with TNP-ATP as the substrate was approximately 20-fold slower with a Vₘₐₓ of 0.010 min⁻¹. The results of these experiments indicated that TNP-ATP can substitute for ATP as a substrate for the autophosphorylation reaction of the EGF receptor protein-tyrosine kinase. Although the Kₘ for the nucleotide analog was similar to that of the authentic substrate, the velocity of the autophosphorylation reaction was dramatically reduced with the TNP-ATP substrate.

TNP-ATP as an Inhibitor of EGF Receptor Protein-tyrosine Kinase Activity—A comparison of the kinetic constants for ATP and TNP-ATP in the autophosphorylation reaction suggested that TNP-ATP might be an effective inhibitor of the ATP-dependent autophosphorylation and substrate phosphorylation reactions catalyzed by the EGF receptor protein-tyrosine kinase. TNP-ATP was first tested as an inhibitor of the autophosphorylation reaction. As shown in Fig. 3A, TNP-ATP appeared to be a competitive inhibitor with respect to ATP in this reaction. Secondary plots of the inhibition data were, however, nonlinear (data not shown), which precluded a simple determination of the Kᵢ for the nucleotide analog. The Kₘ for ATP observed under the conditions of the inhibition experiments (i.e., with 50 mM sodium chloride added) was ~30 μM. Significant inhibition of autophosphorylation activity occurred with TNP-ATP concentrations exceeding 30 μM. These data indicated that although TNP-ATP did support a slow autophosphorylation reaction, it was in effect a competitive inhibitor of the faster, ATP-dependent reaction.

The nucleotide analog was also tested as an inhibitor of the ATP-dependent substrate phosphorylation activity of the EGF receptor protein-tyrosine kinase. In this case, TNP-ATP was seen to be a mixed inhibitor with respect to ATP (see Fig. 3B) and, hence, exerted effects on both the apparent Kₘ for ATP and the apparent Vₘₐₓ. Secondary plots of the inhibition were again nonlinear (data not shown), so that a Kᵢ characterizing the inhibition could not be determined. The Kₘ for ATP in the substrate phosphorylation reaction under the conditions of the inhibition experiment was found to be ~30 μM, and significant inhibition of the reaction occurred with TNP-ATP concentrations exceeding this value. It is possible that the mixed inhibition kinetics observed resulted from a simple competition between ATP and TNP-ATP at the nucleotide binding site combined with a second inhibitory interaction occurring at higher concentrations of the nucleotide analog.

Characterization of the TNP-ATP/Protein-tyrosine Kinase Kinase Activity

**Fig. 1.** Comparison of ATP and TNP-ATP as substrates for the EGF receptor autophosphorylation reaction. A, autophosphorylation kinetics of the recombinant TKD61 protein with [γ-³²P]ATP as substrate. The TKD61 protein was incubated in the presence of 10 mM MnCl₂ and varying concentrations of [γ-³²P]ATP for 10 s at room temperature, and incorporation of ³²P into the TKD61 protein was assayed. Nonlinear least squares fitting of the phosphorylation data yielded values for the Kₘ and Vₘₐₓ of the reaction and the theoretical curve shown. B, autophosphorylation kinetics of the TKD61 protein as assayed by antiphosphotyrosine immunoblotting. The TKD61 protein was incubated as in A, but with unlabeled ATP as substrate. The reaction products were subjected to SDS-PAGE, immunoblotted with the recombinant antiphosphotyrosine reagent RC20, and visualized with enhanced chemiluminescence (ECL) detection (see inset). Relative intensities of the antiphosphotyrosine signals were determined by densitometric analysis of the resulting ECL lumigrams, and the apparent Kₘ value was obtained as above. C, autophosphorylation of TKD61 with TNP-ATP as substrate. Experiment was performed as in B, except that TNP-ATP was substituted for ATP and samples were incubated for 15 min at room temperature. Antiphosphotyrosine was detected by immunoblotting (see inset), and the Kₘ value for TNP-ATP was determined.
Interaction by Fluorescence Spectroscopy—Whereas TNP-ATP is only weakly fluorescent in aqueous solution, the quantum yield of TNP-ATP fluorescence can become significantly enhanced when the nucleotide analog is bound in the hydrophobic environment of an enzyme active site (14, 15). Hence, TNP-ATP has shown great utility as a spectroscopic probe for ATP-dependent enzymes. In the present studies, fluorescence spectroscopy was used to characterize the interaction of TNP-ATP with the EGF receptor TKD. Two recombinant forms of the TKD were examined: the C-terminally complete TKD61 protein described above and the truncated TKD38 protein, which was comprised of residues 645 to 972 of the EGF receptor and therefore lacked the C-terminal autophosphorylation domain present in both the full-length receptor and the TKD61 protein.

The fluorescence emission spectrum of TNP-ATP was recorded in the presence and absence of each of the TKD proteins. In the presence of either the TKD61 protein (Fig. 4A) or the C-terminally truncated TKD38 protein (Fig. 4B), the fluorescence of the nucleotide analog was significantly enhanced, and the wavelength maximum was blue-shifted from 555 to 540 nm. Under the conditions of these experiments, the TNP-ATP nucleotide concentration exceeded that of the added TKD38 or TKD61 protein, so that the enhancement of TNP-ATP fluorescence that occurred upon interaction with the TKD proteins was underestimated. Related experiments indicated that the

![Fig. 2. Velocity of the TNP-ATP-dependent autophosphorylation reaction. A, calibration of the antiphosphotyrosine signal with known quantities of [γ-32P]ATP. The TKD61 protein was incubated with [γ-32P]ATP, and the incorporation of 32P was assayed as described in Fig. 1A. The indicated quantities of labeled TKD61 protein were then subjected to SDS-PAGE, antiphosphotyrosine immunoblotting, and densitometric analysis. The slope of the fitted line indicates the relationship between antiphosphotyrosine signal intensity and phosphotyrosine content. B, the TKD61 protein was incubated at room temperature in the presence of 10 μM MnCl₂ and 50 μM TNP-ATP for the indicated time intervals. Absolute phosphotyrosine content of the TKD61 protein was quantified by immunoblotting and use of the standardization in A. The slope of the fitted line is the velocity of the TNP-ATP-dependent autophosphorylation reaction under the conditions of the assay (0.010 min⁻¹).

![Fig. 3. TNP-ATP as an inhibitor of the ATP-dependent autophosphorylation and substrate phosphorylation reactions of the EGF receptor protein-tyrosine kinase. A, the TKD61 protein was assayed for autophosphorylation activity as described in Fig. 1A in the presence of 10 μM MnCl₂ and varying concentrations of ATP (5–100 μM) and fixed concentrations of the inhibitor TNP-ATP: 0 μM (○), 10 μM (●), 20 μM (▲), 50 μM (▼), and 100 μM (□). Double-reciprocal plots of the velocity data are shown. The velocity data obtained at each TNP-ATP concentration were fit by nonlinear least squares to determine the apparent Kₘ and Vₘₐₓ for ATP, and the lines shown represent the linear transformation of the best-fit hyperbolic curves (see "Experimental Procedures"). B, the TKD61 protein was assayed for substrate phosphorylation activity as in A with the substrate GAT (0.4 gliter) added and incubations of 10 min at room temperature. Assays were performed with varying ATP concentrations and fixed concentrations of TNP-ATP: 0 μM (○), 20 μM (●), 50 μM (▼), 80 μM (▲), and 100 μM (□). The velocity data were analyzed as described in A.
fluorescence emission of TNP-ATP at 540 nm was enhanced approximately 9-fold and 17-fold when the nucleotide was associated with the TKD61 and TKD38 proteins, respectively (data not shown).

Fig. 5A shows the results of titrations in which fixed concentrations of the TKD proteins were treated with increasing concentrations of TNP-ATP and nucleotide fluorescence was monitored. From these spectroscopic data, it was possible to estimate the dissociation constants for the interactions between TNP-ATP and each of the recombinant TKD proteins (see "Experimental Procedures"). Under the conditions of these experiments, TNP-ATP bound with high affinity to both the TKD61 (Kₐ = 0.43 ± 0.22 μM, n = 3) and TKD38 (Kₐ = 54 ± 15 nM, n = 2) proteins. Hence, the truncated TKD38 protein bound TNP-ATP with a significantly higher affinity than did the TKD61 protein.

The affinity of the TNP-ATP/TKD61 interaction was significantly greater than that indicated by the Kₐ for TNP-ATP in the autophosphorylation reaction of TKD61 (see above), which might have been due to the absence of an activating divalent metal ion in the fluorescence assay medium (see "Discussion"). Subsequent titrations were performed in the presence of a minimal concentration (100 μM) of Mn²⁺, sufficient to promote the formation of the Mn⁻²⁻TNP-ATP complex (see Fig. 5B). Higher concentrations of Mn²⁺ were found to cause protein aggregation under the conditions of the fluorescence assays (data not shown). These titrations allowed the determination of the affinities of Mn-TNP-ATP for both the TKD61 (Kₐ = 1.9 ± 1.6 μM, n = 2) and TKD38 (Kₐ = 0.36 ± 0.28 μM, n = 2) proteins. The Kₐ characterizing the Mn-TNP-ATP interaction with TKD61 was similar to the Kₐ for the nucleotide in the TNP-ATP-dependent autophosphorylation reaction of TKD61 (see Table I), which was consistent with the assumption that the Mn-TNP-ATP complex was the active substrate in the autophosphorylation reaction of TKD61.

Fig. 4. Fluorescence analysis of TNP-ATP/EGF receptor TKD interactions. A, the fluorescence emission spectrum of TNP-ATP (1.0 μM) in 20 mM sodium Hepes, 10% (v/v) glycerol, pH 7.4 with (——) or without (-----) added TKD61 protein (0.5 μM). Samples were held at 22 °C, and fluorescence was recorded with excitation at 418 nm and with 8 nm excitation and emission band passes. Each TNP-ATP spectrum shown was corrected by subtraction of an appropriate blank spectrum, that of either a buffer blank or buffer with added TKD61 protein. B, the fluorescence emission spectrum of TNP-ATP (1.0 μM) in the presence (——) or absence (-----) of the TKD38 protein (0.5 μM) recorded as described in A.

Fig. 5. Affinity of TNP-ATP/EGF receptor TKD interactions. A, titration of the EGF receptor TKD proteins with TNP-ATP. Aliquots of TNP-ATP stock solutions were diluted into solutions of either TKD61 or TKD38, each at 0.5 μM, and the fluorescence intensity (excitation 418 × 8 nm; emission 540 × 8 nm) was recorded after each addition. Blank TNP-ATP titrations were performed in an identical manner but without added TKD protein. The fluorescence intensities shown are the differences between TKD61 (●) or TKD38 (○) and blank titration data. Results of three independent titrations of TKD61 and two titrations of TKD38 are included. The titration data were analyzed by curve-fitting to determine the dissociation constant characterizing the interaction and to generate the theoretical curves shown (see "Experimental Procedures"). B, titration of the TKD61 (●) or TKD38 (○) proteins with TNP-ATP in the presence of Mn²⁺. Experiments were performed and analyzed as in A, except that 100 μM MnCl₂ was included in the assay medium to support the formation of the Mn-TNP-ATP complex.
Dissociation and catalytic constants characterizing nucleotide TKD interactions

Dissociation constants ($K_d$) characterizing the binding of TNP-ATP and Mn \cdot ATP complex to the C-terminally complete TKD61 and truncated TKD38 proteins were derived by fitting of Equation 1 to the titration data of Fig. 5. Corresponding $K_d$ values for ATP and Mn \cdot ATP binding to TKD61 were determined by the TNP-ATP competition experiments of Fig. 6. Michaelis-Menton constants ($K_m$) characterizing Mn \cdot ATP and Mn \cdot TNP-ATP as substrates for the autophosphorylation reaction of the TKD61 protein were estimated from the data of Fig. 1. Values are given as the mean \pm S.E. N, number of data sets analyzed in composite by curve-fitting; n, number of independent determinations; ND, not determined.

| Nucleotide        | TKD38 ($K_d$)     | TKD61 ($K_m$)  |
|-------------------|-------------------|---------------|
| TNP-ATP           | 0.054 \pm 0.015 (N = 2) | 0.43 \pm 0.22 (N = 3) |
| Mn \cdot TNP-ATP  | 0.36 \pm 0.18 (N = 2)   | 19 \pm 1.6 (N = 2)    |
| ATP               | ND                 | 47.0 \pm 5.6 (N = 3)  |
| Mn \cdot ATP     | ND                 | 16.1 \pm 2.0 (N = 3)  |
|                  |                    | 5.7 \pm 0.8 (n = 7)   |

The trinitrophenyl analogs of both ATP and GTP have been successfully employed in mechanistic investigations of a variety of nucleotide-dependent enzymes. In the present work, we have explored the potential of TNP-ATP as a substrate analog and spectroscopic probe for protein-tyrosine kinases. The relatively small size of the EGF receptor protein-tyrosine kinase, generated and purified as a recombinant protein, was chosen for these studies. Two forms of the protein-tyrosine kinase were employed here: TKD61, the recombinant cytoplasmic domain of the receptor protein, which contained a functional protein kinase domain and an intact C-terminal autophosphorylation domain; and TKD38, a truncated cytoplasmic domain protein, lacking the C-terminal autophosphorylation domain of the native receptor protein. Although these TKD proteins both lacked the extracellular growth factor binding domain of the EGF receptor, in the presence of the millimolar concentrations of Mn$^{2+}$, they exhibited levels of protein-tyrosine kinase activity similar to that of the purified holoreceptor. These recombinant proteins were also available in quantities sufficient to facilitate spectroscopic studies of their interactions with the fluorescent nucleotide analog.

**DISCUSSION**

Initial experiments demonstrated that TNP-ATP could serve as a functional substrate analog of ATP in the autophosphorylation reaction of the EGF receptor protein-tyrosine kinase. The nucleotide analog supported the autophosphorylation of the TKD61 protein with a concentration dependence similar to that of the authentic nucleotide. However, the maximal velocity of the reaction with TNP-ATP as substrate was approximately (5.6) as well as $K_a$, the dissociation constant for tyrsub binding to the TKD38 protein in the absence of nucleotide (3.8 $\mu$M). The dissociation constant for TNP-ATP binding in the presence of a saturating concentration of tyrsub ($\alpha K_{TNP-ATP}$) determined by the fitting was 0.86 $\mu$M. This value was similar to the dissociation constant characterizing the binding of TNP-ATP to the C-terminally complete TKD61 protein (0.43 $\mu$M, see Table I). Apparently, the peptide substrate, when bound to the active site of the truncated TKD38 protein, mimicked the C-terminal autophosphorylation domain of the TKD61 protein. Occupancy of the peptide binding site of the protein kinase with either the C-terminal autophosphorylation domain in the context of TKD61 or an exogenous peptide substrate in the case of TKD38 resulted in a similar reduction of the affinity of TNP-ATP binding. In related experiments (data not shown), both the peptide substrate GAT and MBP-B3–11, a recombinant fusion protein incorporating sequences from the C-terminal autophosphorylation domain of the ErbB3 protein and previously characterized as a high-affinity substrate for the EGF receptor protein-tyrosine kinase (16), were shown to also attenuate the binding of TNP-ATP to the TKD38 protein.
The observed properties of TNP-ATP as a substrate in the autophosphorylation reaction of the EGF receptor protein-tyrosine kinase suggested that TNP-ATP might be an effective inhibitor of the ATP-dependent reactions of this enzyme. Indeed, TNP-ATP was seen to function as a competitive inhibitor with respect to ATP in the autophosphorylation reaction of the TKD61 protein (see Fig. 3A). TNP-ATP also inhibited the exogenous substrate phosphorylation activity of TKD61, showing mixed-type inhibition kinetics with respect to the substrate ATP (see Fig. 3B). Although no simple mechanism can be forwarded to explain quantitatively the details of the observed TNP-ATP inhibition kinetics, the kinetics were consistent with a competitive interaction between TNP-ATP and ATP at the catalytic site, in conjunction with a second, lower-affinity inhibitory interaction between the TNP-ATP and the TKD61 protein.

Our investigations of the substrate activities and inhibitor properties of TNP-ATP suggested that the nucleotide analog bound with high affinity to the catalytic site of the EGF receptor protein-tyrosine kinase. Given that the TNP-ATP molecule exhibits an enhanced fluorescence in hydrophobic environments (19), fluorescence spectroscopy was used to monitor the binding of the nucleotide analog to the recombinant EGF receptor TKD. Initial experiments indicated that the fluorescence of TNP-ATP was significantly enhanced in the presence of micromolar concentrations of the TKD61 protein. A subsequent titration revealed an interaction with a dissociation constant of 0.43 μM. The truncated TKD38 protein, which lacked the C-terminal autophosphorylation domain of the EGF receptor, bound the TNP-ATP molecule with an even greater affinity (Kₐ = 54 nm) than did the full-length TKD61 protein.

The Kₐ values for TNP-ATP binding to the TKD proteins were significantly lower than the Kₐ for ATP observed in the autophosphorylation studies. As the autophosphorylation assay medium included 10 mM MnCl₂, it was considered that the true substrate of the TNP-ATP-dependent autophosphorylation reaction was the Mn²⁺ complex of TNP-ATP (MnTNP-ATP). Subsequent TNP-ATP titrations were therefore performed with Mn²⁺ present in the assay medium, at

Fig. 6. Effects of ATP upon the fluorescence of the TNP-ATP/TKD61 complex. A, as described in Fig. 5, a solution of TNP-ATP (0.1 μM) and TKD61 (1.0 μM) was prepared, and the fluorescence of TNP-ATP was recorded as ATP was added to increasing concentrations. The fluorescence intensity data were corrected by subtracting the contributions of the TKD61 protein and free TNP-ATP and hence represent the enhancement of fluorescence occurring with the TNP-ATP/TKD61 interaction. Results of three independent experiments are included. The data were analyzed as described under “Experimental Procedures” to yield estimates for the dissociation constant for ATP binding (see Table I) and the best-fit theoretical curve shown. B, effect of MnCl₂ on the fluorescence of the Mn⁺TNP-ATP/TKD61 complex. Titrations were performed and analyzed as in A, except that a TNP-ATP concentration of 1.0 μM was employed. Increasing concentrations of MnCl₂ and ATP were added, so that the concentration of free Mn²⁺ was held constant at 50 μM as the Mn⁺ATP concentration was increased. Concentrations of Mn⁺ATP complex and free Mn²⁺ were calculated as described (13). Results of three independent experiments are included.

Fig. 7. Effect of a peptide substrate on the affinity of the TNP-ATP/TKD38 interaction. The TKD38 protein (0.5 μM) was titrated with TNP-ATP in the presence of various fixed concentrations of the peptide tyrsub, and the fluorescence intensity was recorded after each TNP-ATP addition as described in Fig. 5. Shown are titrations performed in the absence () or presence () of 20 μM tyrsub. For each tyrsub concentration, the apparent dissociation constant for TNP-ATP binding (KₐTNP-ATP) was determined by fitting of Equation 1, and the theoretical curves shown were generated. The inset shows the dependence of KₐTNP-ATP upon the concentration of tyrsub and the theoretical curve yielded by fitting of Equation 2 (see “Experimental Procedures”).
concentrations sufficient to promote the formation of the Mn-TNP-ATP complex. From these experiments it was possible to estimate the dissociation constants for Mn-TNP-ATP binding to TKD38 (K_d = 0.36 μM) and TKD61 (K_d = 1.9 μM). The latter dissociation constant was similar to the K_d value for TNP-ATP in the autophosphorylation reaction (see Table I). As was the case for the free TNP-ATP molecule, the Mn-TNP-ATP complex bound with significantly higher affinity to the TKD38 protein than to the TKD61 protein.

Given these observations, it hypothesized that the C-terminal autophosphorylation domain of the TKD61 protein, which must at least transiently occupy the peptide substrate binding site of the enzyme, could modulate the binding of nucleotide substrates at the active site. This hypothesis predicted that peptide substrates of the EGF receptor-protein-tyrosine kinase would in the context of the TKD38 protein also modulate the binding of the TNP-ATP nucleotide. The peptide substrate, tyrsub, was found to significantly reduce the affinity of binding of TNP-ATP to the TKD38 protein (see Fig. 7). In the presence of a saturating concentration of tyrsub, the K_d for TNP-ATP binding to TKD38 was increased by a factor of 5.6 and assumed a value of 0.86 μM, similar to the K_d for TNP-ATP binding to the C-terminally complete TKD61 protein (0.43 μM). It was therefore considered that the peptide substrate, when bound to the TKD38 protein, mimicked the C-terminal autophosphorylation domain of the TKD61 protein and exerted a similar negative effect on nucleotide binding affinity. Implicit in this interpretation of the data is the assumption that the C-terminal domain of the TKD61 protein occupied the peptide binding site at equilibrium.

The negative effects of both tyrsub and the C-terminal autophosphorylation domain on the binding of TNP-ATP to the TKD proteins appeared not to result from a direct competition between the peptide substrates and the nucleotide for a single binding site, as the presence of a saturating concentration of tyrsub did not abolish nucleotide binding, but instead resulted in only a finite change in the nucleotide dissociation constant (see Fig. 7, inset). Apparently, nucleotide and peptide substrate could simultaneously bind to the kinase domain catalytic site, as a steady-state kinetics study of the EGF receptor protein-tyrosine kinase has previously indicated (10). This study, in which the concentrations of ATP and peptide substrate were co-varied, also indicated a negative interaction between nucleotide and peptide binding sites. In the absence of EGF-stimulation, occupancy of the nucleotide binding site by ATP led to a 20-fold increase in the K_m of the peptide substrate. Interestingly, the EGF-stimulated receptor showed a much weaker interaction between the peptide and nucleotide binding sites, which resulted in an effective lowering of the K_m for the peptide substrate in the presence of saturating concentrations of ATP. As the fluorescence experiments described here were performed under nonactivating conditions, i.e. in the absence of millimolar concentrations of an activating divalent metal ion, our observation of a negative interaction between nucleotide and peptide binding sites was consistent with these previous steady-state kinetics studies.

The published three-dimensional structures of the kinase domain of the cAMP-dependent protein kinase obtained in the presence and absence of a high affinity peptide substrate indeed indicate that peptide substrates occlude the ATP binding site and could certainly modulate the nucleotide-binding properties of this enzyme (20). The recently obtained three-dimensional structure of the insulin receptor protein-tyrosine kinase domain (21) shows a general structural similarity to that of the cAMP-dependent protein kinase, which suggests that most protein kinase subunits share basic structural and functional elements. Our hypothesis that the C-terminal autophosphorylation domain of EGF receptor protein modulates the nucleotide-binding properties of TKD is therefore consistent with the presently available structural data. An important remaining question is whether the observed interactions between the C-terminal autophosphorylation domain of the EGF receptor and the active site are intramolecular or intermolecular in nature. Certainly, as the transphosphorylation of wild-type and kinase-deficient EGF receptor proteins has been documented (22), such intermolecular interactions between EGF receptor proteins would be reasonable to assume. This question could be addressed by investigating the TNP-ATP binding properties of the TKD38 protein in the presence of a mutant TKD61 protein that is devoid of nucleotide binding activity.

In summary, we have demonstrated that the fluorescent nucleotide analog TNP-ATP was a functional substrate for the EGF receptor protein-tyrosine kinase. The analog was seen to be an inhibitor of the ATP-dependent reactions catalyzed by the protein-tyrosine kinase, apparently competing with ATP for occupancy of the nucleotide binding site. The binding of TNP-ATP to recombinant EGF receptor TKD proteins could be directly characterized by fluorescence spectroscopy. The presence of the C-terminal autophosphorylation domain was shown to reduce the affinity of binding of the nucleotide analog. It was hypothesized that the C-terminal autophosphorylation domain of the EGF receptor occupied the peptide substrate binding site of the TKD at equilibrium and lowered the affinity of the nucleotide binding site for TNP-ATP. TNP-ATP was therefore seen to be a useful spectroscopic probe for studying the structure and function of the EGF receptor protein-tyrosine kinase, and would likely be of great utility in examinations of other protein-tyrosine kinases.

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