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Fluorescent and colored trinitrophenylated analogs of ATP and GTP

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REVIEW ARTICLE

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Abbreviations: TNP-nucleotides, 2',3'-O-(2,4,6-trinitrocyclohexadienylidene)-derivatives of nucleotides; AMP-PNP and GMP-PNP, adenylyl- and guanylyl-imidodiphosphates, respectively; AMP-PCP, adenylyl-(β,γ-methylene)-diphosphate; EnvZ and CheA, osmosensor and chemotaxis sensor histidine protein kinases, respectively; CFTR, cystic fibrosis transmembrane conductance regulator; Pgp, P-glycoprotein; SV40, simian virus 40; HIV-1, human immunodeficiency virus 1; RT, reverse transcriptase; CF_1, catalytic portion of the chloroplast ATP synthase; PGK, 3-phosphoglycerate kinase; PRK, phosphoribulokinase; WNDP, Wilson's disease protein; 3PG, 3-phospho-D-glycerate; NBF, nucleotide binding fold; FRET, fluorescence resonance energy transfer.

Fluorescent and colored trinitrophenylated (TNP) analogs of ATP and GTP can interact with nucleotide-requiring enzymes and proteins as a substitute for the parent nucleotide. These analogs have strong binding affinities for most nucleotide-requiring systems. Their bindings are easily detected by absorption and fluorescence changes in the visible region. Recent years have seen dramatic developments in the application of the TNP nucleotide analogs as spectroscopic probes for the study on the nucleotide-interacting properties of various enzymes and proteins including their mutants. This review is intended as a broad overview of currently extensively used applications of the nucleotide analogs in various biological systems.

Keywords: TNP-ATP, TNP-GTP, trinitrophenylated ATP, trinitrophenylated GTP, fluorescent nucleotide analogs, nucleotide-requiring proteins.

Running title: Fluorescent nucleotides, TNP-ATP and TNP-GTP

Nucleoside triphosphates are crucial mediators of life. ATP is used to drive unfavorable chemical reactions, to fuel biological machines, and to regulate a number of processes via protein-phosphorylation. GTP, in turn, is used almost exclusively for the regulation of signal transduction and transport processes. Proteins that bind and use ATP and GTP for enzymatic reaction and regulation are very diverse [1].

Fluorescence is a powerful technique to obtain information about the size and structure of proteins, allows quantitation of the kinetic and equilibrium constants describing the systems. Using a fluorescence microscope, it can also shed light on the cellular distribution of the proteins. One of the primary reasons for the widespread use of fluorescence to study proteins is the inherent high sensitivity of the method. Thus considerable effort has been expended on modifying nucleotides to improve their utility as fluorescent probes for investigations of nucleotide-binding proteins [2-5]. Rendering the nucleotide fluorescent, while retaining the biological activity of the parent nucleotide, can provide useful information about interactions of nucleotide with protein.

Various fluorescent nucleotide analogs including those with modified base, phosphate, and ribose moieties have been developed (for recent reviews see [4, 5]). The first fluorescent ribose-modified ATP appears to have been 2’,3’-O-(2,4,6-trinitrocyclohexadienylidene) adenosine 5’-triphosphate (TNP-ATP) introduced by Hiratsuka and Uchida in 1973 [6]. The corresponding analog of GTP (TNP-GTP) was synthesized by Hiratsuka 12 years later [7].

These colored fluorescent nucleotide analogs can be excited at wavelengths (408 and 470 nm) far from where proteins or nucleotides absorb, and fluoresce at 530-560 nm [7-9]. It should be emphasized that they are weakly fluorescent in aqueous solutions, while the fluorescence can be enhanced markedly upon binding to a protein. This property enables us to use the analog as a fluorescent probe in investigations of binding interactions of nucleotide with various proteins. Techniques employing the TNP nucleotide analogs have proved to be complementary to, and in several cases even superior to, the traditional-radionucleotide based

techniques. Increasing costs and public concerns associated with radioactive isotope use and dispersal are also making the use of TNP nucleotide analogs more attractive in research use.

The TNP nucleotide analogs are prepared by an easy one-step synthesis [6-8] and are commercially available. Within the past 15 years, over 400 papers describing their use have been published. Such applications of TNP nucleotide analogs have helped to clarify the structure-function relationships of numerous nucleotide-requiring enzymes and proteins. Especially, there have recently been a growing number of papers describing their use as a simple and reliable test for the assessment of the nucleotide-binding capacity of various mutant proteins. An attempt is made in this review to be comprehensive and critical in assessing the recent applications of TNP-ATP and TNP-GTP to biological systems.

STRUCTURES

The ribose moiety of ATP is easily trinitrophenylated by 2,4,6-trinitrobenzenesulfonate at pH 9.5 in aqueous solution to form the Meisenheimer spiro complex [6, 8]. The corresponding analog of GTP is also obtained under similar conditions with the use of 2,4,6-trinitrochlorobenzene [7]. Figure 1 shows the structures of the TNP nucleotide analogs at neutral or basic pH values. The proton NMR spectrum of TNP-ATP showed that the H-8 resonance signal is shifted upfield in comparison with that of ATP [6, 10], indicating interaction between this region of the adenine base and a part of the TNP moiety. Such a proximity of the two moieties of TNP-ATP was clearly shown by the X-ray crystal structure of TNP-ATP bound to the histidine protein kinase CheA [11] (see Fig. 2 in the section on APPLICATIONS). Acidification of TNP-ATP under mild conditions results in the opening of the dioxolane ring at the 2’-oxygen to yield the 3’-O-TNP derivative as the only product [12].

SPECTROSCOPIC PROPERTIES

At neutral or basic pH values, TNP nucleotide analogs show two visible absorption maxima at 408 and 470 nm, assuming a bright orange color. These two maxima are characteristic of Meisenheimer addition complex such as 1-ethoxy-2,4,6-trinitroanisole [6]. On the other hand, TNP nucleotide analogs in water show a single fluorescence emission maximum at 561 nm upon excitation with light in the 410 or 460 nm regions. As the pH is decreased, either visible absorption or fluorescence of them is gradually decreased. The pKa value of 5.2 obtained by the spectrophotometric pH titrations of TNP-ATP is identical with that obtained by the fluorometric pH titrations [8]. Thus only the Meisenheimer spiro complex forms of TNP nucleotide analog (Fig. 1) show both the visible absorption and fluorescence.

To be used as a spectroscopic environmental probe for proteins, the molecule must be sensitive to some indicator of local environment, e.g., polarity and viscosity. Wavelengths of visible absorption maxima of TNP-ATP depend on solvent polarity [8, 9]. For example, they vary between 408 nm in water and 410 nm in 80 % ethylene glycol for the first maximum as well as between 470 in water and 474 nm in 80 % ethylene glycol for the second maximum. The position of the fluorescence emission maximum of TNP-ATP varies more significantly with solvent [8, 10]. For example, it is at 561 nm in water and at 533 nm in *N*,*N*-dimethylformamide. On the other hand, the quantum yield is enhanced 75-fold in going from water to this organic solvent where the absolute quantum yield is 0.015. It should be noted that both the intensity and the maximum of the emission spectrum change gradually with change of the composition of the solvent, and there is no significant change in the shape of the emission spectrum. The solvent polarity has been expressed using Kosower's empirical polarity *Z* scale [13]. Both the location of the emission maximum and the emission quantum yield of TNP-ATP showed very good correlation with the *Z* value [8]. The fluorescence of TNP-ATP is also sensitive to changes in solvent viscosity. The quantum yield is increased 3.7-fold in going from 0 to 30 % sucrose at 25 °C. At the same time, the

wavelength of emission maximum is decreased from 561 to 547 nm.

These fluorescence properties of TNP-ATP, together with its visible absorption properties, make it possible to use TNP nucleotide analogs not only as fluorescent but also as chromophoric probes for nucleotide-requiring enzymes and proteins. The spectroscopic properties of TNP nucleotide analogs are independent of structures of base and phosphate moieties of parent nucleotides. Thus there is no significant difference in spectroscopic properties between TNP-ATP and TNP-GTP [7]. Furthermore, it is impossible to monitor the enzymatic hydrolysis of the TNP-nucleoside triphosphates spectrophotometrically.

APPLICATIONS

TNP-ATP was synthesized as a chromophoric [6, 10] and fluorescent [9, 14] probe to obtain information about the environment around the ATP binding site of the myosin ATPase, the best-known example of motor proteins. TNP-ATP was hydrolyzed by the myosin ATPase. Upon binding to myosin, fluorescence of TNP-ATP and TNP-ADP was markedly enhanced. These reports have extended the use of TNP nucleotide analogs to other numerous enzymes and proteins. Table 1 lists recent selected applications of TNP nucleotide analogs with some of their fluorescent and biological characteristics in various biological systems. The most remarkable in their recent applications is the use as a simple and reliable test for the assessment of nucleotide-interacting properties of mutant proteins. Furthermore, the applications of TNP nucleotide analogs have been extended to those coupled with fluorescence microscopy and X-ray crystallography.

Kinetic studies

The most extensive applications of TNP nucleotide analogs to date have been in kinetic and equilibrium measurements of the interaction of nucleotides with enzymes and proteins.

These methods generally involve the study of fluorescence or absorption changes associated with binding and dissociation of TNP nucleotide analogs as substitutes for the natural nucleotides. It should be emphasized that most enzymes and proteins bind TNP nucleotide analogs stoichiometrically and approximately from one to three orders of magnitude more tightly than the natural nucleotides with dissociation constants of 0.3-50 μM. At the same time, increases in fluorescence of the bound TNP nucleotide analogs (2-12-fold) are observed in various systems (Table 1).

F-type ATPases are involved in ATP synthesis in eubacteria, mitochondria and chloroplasts (e.g. F_1-ATPase). P-type ATPases are cation pumping ATPases (e.g. Na^+,K^+-, H^+,K^+-, and Ca^{2+}-ATPases). The most extensive use of TNP nucleotide analogs to date has been in studies on these two ATPase families [15-17]. Recently, the analogs have been also used in studies on the traffic ATPases (ABC transporters) including CFTR and Pgp, large family of membrane-associated export and import systems. TNP-ATP and TNP-GTP bind to CFTR with high affinities [18-20]. Pgp can hydrolyze TNP-ATP but at a much slower rate than ATP [21, 22].

TNP-ATP was also used for studies on various kinases. TNP-ATP acted as a substrate for PRK [23]. However, for mevalonate kinase [24] and PGK [25, 26], this analog was not a substrate but a strong competitive inhibitor toward ATP and ADP.

TNP nucleotide analogs are suitable flurescent probes to study the nucleotide binding properties of ATP-dependent DNA helicases, which play essential roles in replication, repair, recombination and transcription of DNA. They include DnaB [27] and SV40 T antigen [28]. Both proteins bind TNP-ATP and TNP-ADP stoichiometricaly with high affinities. DnaB hydrolyzes TNP-ATP at a rate similar to that of dATP whereas SV40 T antigen is unable to hydrolyze it. With the aid of these TNP nucleotide analogs, it was revealed that the nucleotide binding specificity of the T antigen is similar to that of DnaB.

P2X receptors are membrane ion channels that open in response to the binding of

extracellular ATP. There are seven genes in vertebrates encode P2X receptor subunits (for recent reviews see [29, 30]. Except for the F- and P-type ATPases, the most extensive use of TNP nucleotide analogs has been in studies on the interactions with P2X receptors. TNP-ATP is strongly selective for receptors containing $P2X_1$ and $P2X_3$ subunits as an antagonist [31]. The IC_{50} (50 % inhibitory concentration) is about 1 nM. At present, TNP-ATP is a useful tool for identifying the participation of these receptor subunits. Within the past 4 years, over 70 papers describing such a use have been published.

Recently, the first evidence of direct binding of ATP to cytosolic domains of the pore-forming subunits of ATP-sensitive K^+ channels has been obtained from the study with an extensive use of TNP-ATP [32]. It had been proposed that ATP regulation of the channel activity may involve direct binding to the pore-forming inward rectifier subunit despite the lack of known nucleotide-binding motifs. TNP-ATP was found to bind to the COOH termini, but not the NH_2 ones, of the subunits of ATP-sensitive K^+ channels. The kinetic analysis of TNP-ATP binding suggested that the COOH termini have a single nucleotide-binding site.

Annexin VI is a 68-kDa calcium-, phospholipid-, and cytoskeleton-binding protein. This protein binds not only TNP-ATP but also TNP-GTP with high affinities [33]. It was revealed that annexin VI is a GTP-binding protein and the binding of GTP may have a regulatory impact on the interaction with membrane.

Ligand binding studies

In case a ligand competes with the TNP nucleotide analog for the binding site on protein, the binding affinity of the ligand can be measured from spectral changes originated from the bound TNP analog. In these experiments, fluorescence and absorption titrations of protein with the TNP analog are first carried out, and then the bound analog is displaced by increasing concentrations of ligand added, which is monitored by a decrease in the fluorescence or absorption. Alternatively, protein is titrated with the TNP nucleotide analog in the absence

and presence of varying, fixed concentrations of the ligand of interest. The presence of ligand as competitor has profound effects on the binding of TNP analog, making it progressively more difficult to saturate the protein in the presence of higher concentrations of the ligand. Using either experiment of the displacement or the competition, the binding affinity of the ligand of interest can be measured. A detailed account of such methods is beyond the scope of this review and the reader is referred to the literatures [34, 35]. Using these methods, the ligand binding affinities for enzymes and proteins not only of natural nucleotides and their non-fluorescent analogs but also various biological compounds have been measured as described below.

Binding affinities of natural nucleotides to the Ca^{2+}-ATPase were measured using TNP-ATP and TNP-ADP as probes [15]. The second nucleotide-binding sites (NBF2) of CFTR can bind not only ATP and TNP-ATP, but also GTP and TNP-GTP [20]. For EnvZ, which is a histidine protein kinase important for osmoregulation in bacteria, the binding affinities of ATP and ADP were measured using TNP-ADP [35].

TNP-ATP was utilized to quantify the affinity for HIV-1 RT, an RNA-dependent DNA polymerase that transcribes the viral RNA into a double-strand DNA. The binding affinities of oligonucleotide primers with varying size lengths were easily measured with the aid of changes in fluorescence emitted from the bound TNP-ATP [34].

Interestingly, phosphatidylinositol phopholipids compete for TNP-ATP binding to the COOH termini of ATP-sensitive K^+ channels [36]. From the displacement experiments, it was suggested that the COOH termini of the channels form a nucleotide- and phopholipid-modulated channel gate on which ATP and phopholipids compete for binding.

Wilson's disease is caused by mutations in gene encoding a copper-transporting ATPase (Wilson's disease protein, WNDP). The Lys^{1010}-Lys^{1325} fragment of the protein where numerous mutations had been identified was overexpressed, purified, shown to form an independently folded ATP-binding domain. TNP-ATP binds to this fragment more tightly

than ATP [37].

Energy transfer studies

The technique of FRET provides a means of estimating the distance between a fluorescence donor and an acceptor, and has been used to determine the distance between several specific sites in proteins. The TNP nucleotide analog is a potentially valuable fluorescence acceptor because the wide range of wavelengths over which it absorbs conveniently overlaps the emission spectra of many commonly used fluorescence donors. Thus, TNP nucleotide analogs have been extensively used in the FRET studies with various enzymes and proteins.

For the Na^+,K^+-ATPase, the distance between the donor 5'-(iodoacetamido) fluorescein attached to Cys457 and the acceptor TNP-ATP bound to the active site was measured [16]. Interestingly, the distance (25 Å) was shown to increase 3 Å when the enzyme changes from the Na^+ to the K^+ conformation.

The most extensive use of TNP nucleotide analogs in the FRET measurements has been in the studies on CF_1 containing five different subunits designated α–ε in order of decreasing molecular weight. The distance between the donor *N*-(1-pyrenyl)maleimide attached to Cys63 on the N-terminal domain of β subunit and the acceptor TNP-ADP at the nucleotide binding site was measured to be 42 Å [17]. Since binding of ADP to the β subunit caused an increase in the fluorescence intensity of the donor, the nucleotide binding domain and the N-terminal domain of the β subunit were suggested to communicate with each other over a significant distance via conformational changes.

PRK forms a stable ternary complex with TNP-ATP at the active site and an allosteric activator NADH [23]. Using the former as a fluorescence acceptor and the later as a fluorescence donor, the distance between the two sites was estimated as 21 Å.

Binding of TNP-GTP to tubulin caused a large increase in the analog fluorescence [38]. This fluorescence increase disappeared completely when excess GTP was added, indicating

that TNP-GTP binds to the exchangeable GTP binding site. It was shown that 0.75 mol of the analog was bound per mol of the protein. Electron micrographs of TNP-GTP•tubulin polymerized by paclitaxel (Taxol) showed normal microtubules. The distance between Taxol at the drug binding sites and TNP-GTP at the exchangeable GTP binding sites on tubulin polymers was measured to be about 16 Å. However, no FRET was observed between a ligand bound to the colchicine sites and the bound TNP-GTP, indicating that the colchicine sites and exchangeable GTP binding sites are at least 40 Å apart.

X-Ray crystallography

Prior to the X-ray crystallographic analysis of a ligand-protein complex, it is often required to know the ligand-binding properties of the protein in the crystal form. For PGK, the use of TNP-ATP made it possible to determine binding constants for the nucleotide substrates even in the crystal forms [26]. A displacement of TNP-ATP bound to two different crystals of the enzyme, the binary complex with 3PG and the ternary complex with 3PG and AMP-PCP, was monitored upon incubation with ADP or ATP using single-crystal microspectrophotometry. In comparison with solution [25], stronger binding of the nucleotides could be detected in the presence of 3PG in both types of crystals. This result indicated that the antagonistic substrate binding, characteristic of the enzyme in solution, is not retained in the crystal forms.

TNP-ATP inhibits phosphorylation of the bacterial histidine protein kinase CheA by competing with ATP [39]. TNP-ATP is not hydrolyzed by CheA even though the enzyme binds this analog approximately three orders of magnitude more tightly than ATP. The X-ray crystal structures of CheA in complex with TNP-ATP and AMP-PCP have recently been solved [11] and illustrated a different mode of binding for TNP-ATP (Fig.2). In the structures, TNP-ATP and AMP-PCP have similar placement of the adenine base in the hydrophobic cleft. However, the ribose of TNP-ATP adopts an orientation that promotes interaction between the TNP moiety and hydrophobic (I454, I459, and L486) and hydrophilic

(K458 and K462) side chains. This placement of the ribose projects the three phosphates into a more solved-exposed position relative to AMP-PCP. As a consequence the position of the TNP-ATP phosphate is far from the Mg^{2+}-coordinating H405 and N409, resulting in that the residue still hydrogen-bonding to the TNP-ATP phosphates is H413 alone. This explains well the Mg^{2+}-independent binding of TNP-ATP and the inability of CheA to hydrolyze TNP-ATP [39]. The interaction of the TNP moiety may be exploited for designing CheA-targeted drugs that would not interfere with host ATPases.

Microscopy

Several groups have reported the use of TNP-ATP and TNP-GTP coupled with microscopy to greatly enhance the sensitivity of the observations. The TNP nucleotide analogs possess the useful characteristic of exhibiting greatly enhanced fluorescence when bound to proteins, thus greatly reducing the problem of background fluorescence upon observations, especially under epifluorescence illuminations.

The first nucleotide binding fold (NBF1) of CFTR and its disease-causing mutant form were expressed in fusion with the maltose-binding protein and used to check their abilities of interactions with TNP-ATP [19]. TNP-ATP was found to bind similarly to both the wild type and mutant fusion proteins. ATP effectively displaced all of the bound TNP-ATP, indicating that the site involved is capable of binding of the natural substrate. By confocal fluorescence imaging, TNP-ATP was shown to bind throughout organized fibrous networks of both the wild type and mutant fusion proteins, indicating that each fusion proteins within the network had retained the capacity to bind nucleotide.

Using TNP-ATP, real-time fluorescence imaging of extracellular ATP binding sites on inner and outer hair cells isoltated from the guinea pig organ Corti was achieved by epifluorescence microscopy [40]. Suramin, a nonselective P_2 purinoceptor antagonist reduced the fluorescence emitted from the bound TNP-ATP, indicating that the binding sites on the cells

are P_2 receptors. Binding of TNP-ATP to P_2 receptors was also confirmed by its antagonism of the inward current elicited by ATP in voltage-clamped hair cells.

CONCLUSIONS

I have shown that TNP-ATP and TNP-GTP mimic the binding characteristics of ATP and GTP, respectively, in their interactions with various enzymes and proteins. Their spectroscopic properties make them valuable tools with which to determine the kinetic parameters of nucleotide-protein interactions. Furthermore, they act as potentially valuable fluorescence acceptors in FRET experiments. Recently, their applications have been extended not only to the use as a simple and reliable test for the assessment of nucleotide-interacting properties of mutant proteins but also to that coupled with X-ray crystallography and fluorescence microscopy. Thus, TNP nucleotide analogs serve as powerful tools for studies on the nucleotide-requiring biological systems.

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Legends for Figures

Fig. 1. Structures of TNP-ATP and TNP-GTP at neutral or basic pH values. At acidic pH, the opening of the dioxolane ring of TNP-ribose moiety occurs at 2'-oxygen to yield 3'-O-(2,4,6-trinitrophenyl) derivative as the only product [12].

Fig. 2. Mg^{2+}•AMP-PCP (A) and TNP-ATP (B) bound to CheA. Nucleotide analogs and side chains involved in nucleotide binding are shown in ball and stick, and sticks, respectively. In the complex with AMP-PCP (A), Mg^{2+} (light green), H405 (pink), N409 (green), and H413 (magenta) interact with the phosphate moiety. Residues involved in the interaction with the TNP moiety (yellow) are I454 (blue), I459 (cyan), L486 (purple), K458 (dark green), and K462 (dark red). Coordinates of 1i58 and 1i5d in the Brookhaven Protein Data Bank were used in (A) and (B), respectively [11].

Table 1. Parameters of TNP nucleotide binding to proteins.

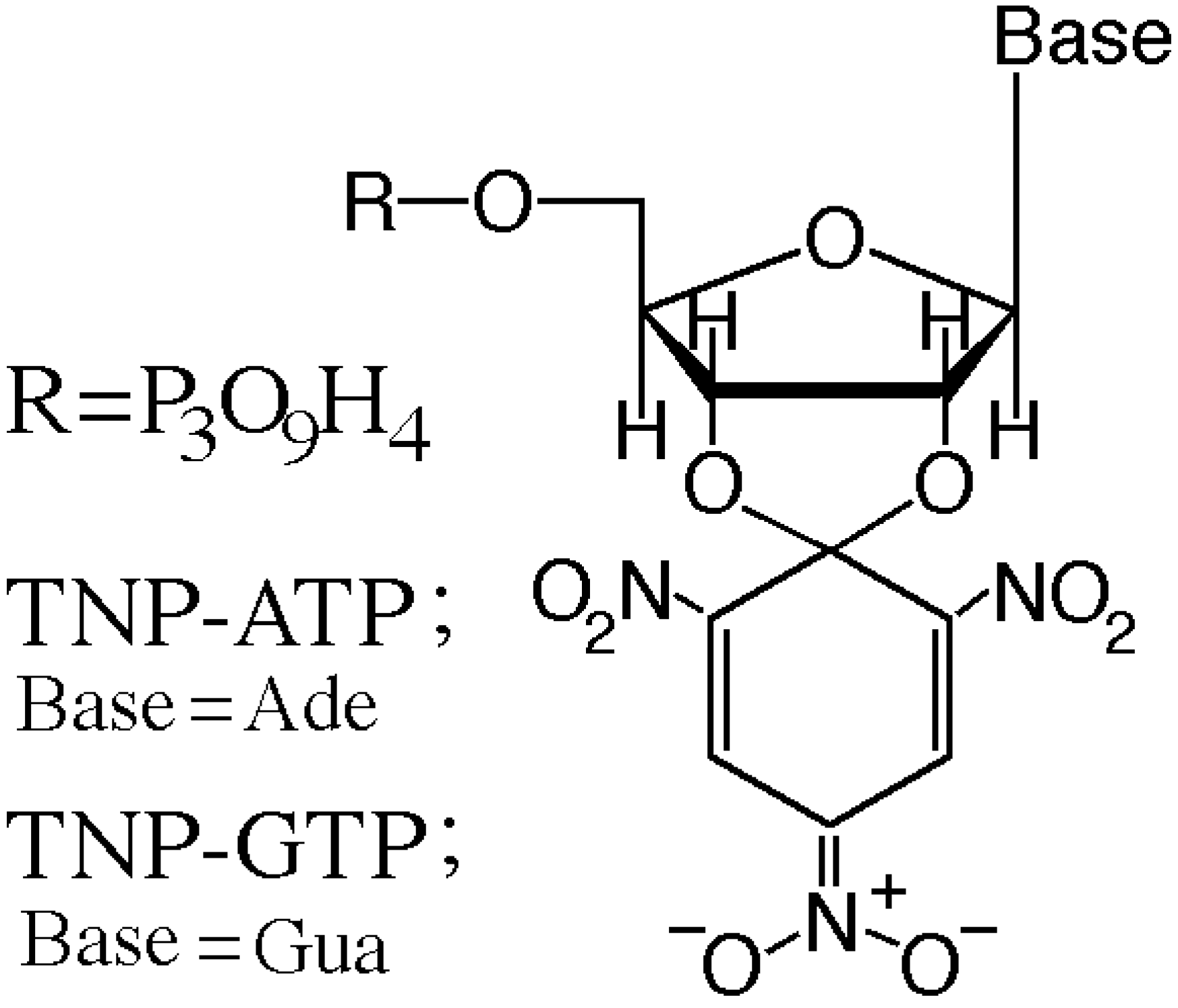
Protein	TNP-derivative	n[a]	Subst.[c]	K_d(μM)[d]	ΔF[e]	Application[f]	Ref
Ca^{2+}-ATPase	ATP		+	0.35 (30)		LB	15
(Lys^{329}-Phe^{740} loop)	ATP	0.85		1.9 (250)	3-12		
Na^+,K^+-ATPase	ATP		–			FRET	16
CF_1	ADP			0.5-1 (46)		FRET	17
CFTR	ATP	1.1		0.81	10	LB	18
(NBF1)	ATP	1		1.8 (1.8)		MS	19
(NBF2)	ATP			22		LB	20
	GTP			3.9 (33)			
Pgp	ATP	2	+	43-50(404-460)	4-5	KN	21
	ADP	2		42 (407)		KN	22
PRK	ATP	0.84	+		6	FRET	23
mevalonate kinase	ATP	0.9	–	12 (19)	6	KN	24
PGK (solution)	ATP	1	–	9.5 (270)	10	KN	25
(crystal)	ATP	1		29 (210)		XR	26
EnvZ	ATP			1.9 (60)		LB	35
	ADP			3 (300)	3		
Che A	ATP	2.1	–	K_d1 = 0.5 (260)	5	LB	39
				K_d2 = 1.7(1100)		XR	11
DnaB	ATP	1[b]	+	1.6		KN	27
	ADP			0.5 (1)			
SV40 T antigen	ATP	0.89[b]	–	0.35	8.7	KN	28
	ADP	0.98[b]		2.6 (12)	8.8		
HIV-1 RT	ATP			21	2	LB	34
P2X receptors	ATP					KN	29-31
	ATP					MS	40
ATP-sensitive K^+ channels	ATP	0.89		2.6		KN	32
	ATP	0.36		0.89	4.7	LB	36

Table 1. (continued)

Protein	TNP-derivative	n[a]	Subst.[c]	K_d(μM)[d]	ΔF[e]	Application[f]	Ref
Annexin VI	GTP	1.05		1.3	5.5	KN	33
WNDP (Lys^{1010}-Lys^{1325} fragment)	ATP			1.9 (268)		LB	37
tubulin	GTP	0.75				FRET	38

[a]The binding stoichiometry (mols of TNP nucleotide analog per mol of protein). [b]Per monomeric protein. [c]Active (+) or inactive (–) as a substrate. [d]Dissociation constants for the TNP nucleotide analog and the corresponding natural nucleotide (in parentheses). [e]The ratio of the fluorescence intensity of bound analog to that of unbound one. [f]KN, LB, FRET, MS, and XR represent studies of kinetics, ligand binding, fluorescence resonance energy transfer, microscopy, and X-ray crystallography, respectively.

Fig. 1



$R=P_3O_9H_4$

TNP-ATP;
Base = Ade

TNP-GTP;
Base = Gua

Fig. 2