[18] Fluorescent Nucleotide Analogs: Synthesis and Applications

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Introduction

Nucleoside 5′-triphosphatases are involved in many important cellular processes such as energy transduction in molecular motors, ion transport, and signal transmission, as well as in the topological processing of nucleic acids and the fidelity of protein synthesis. One of the primary reasons for the widespread use of fluorescence to study nucleotide-binding proteins is the inherent high sensitivity of the method—fluorophores with high extinction coefficients, good quantum yields (i.e., >0.1), and absorption bands at longer wavelengths than endogenous backgrounds [e.g., mid-ultraviolet (UV) and longer] can be readily detected at submicromolar concentrations. Fluorescence can provide information about the size and structure of the proteins, allows quantification of the kinetic and equilibrium constants describing the system, and can also shed light on the cellular distribution of the proteins. Intrinsic protein fluorescence, primarily from tryptophan and tyrosine residues, has been used to obtain detailed information about many of these parameters but there are significant advantages in studying nucleotide fluorescence. Specifically, if the nucleotide itself can be spectroscopically isolated, then a unique fluorophore, which can be localized to a defined position, exists in the system.

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The intrinsic fluorescence of the common nucleotides, at ambient temperatures and neutral pH, is far too low (quantum yields on the order of 10^{-4})[1] to be of general use in the investigation of nucleotide-binding proteins, especially since the longest absorption maxima of nucleotides are near 260 nm, which overlaps with intrinsic protein absorption. Some naturally occurring modified nucleosides, including 4-thiouridine, 7-methylguanosine, N^6-acetylcytidine, and the Wye derivatives (formerly known as the Y bases) are fluorescent[2] and have been useful as probes of tRNA but have found few applications outside of this area.

Considerable effort has been expended on modifying nucleotides to improve their utility as fluorescent probes. Among the first such modified nucleotides were the "etheno" series synthesized by Leonard and collaborators.[2,3] The most commonly used nucleotide in this class is the 1,N^6-ethenoadenosine derivative, εATP (**I** in Fig. 1). Leonard has written an excellent review of the use of etheno-bridged nucleotides in enzyme reactions and protein-binding studies.[4] More conservative changes to the nucleotide base in formycin 5′-triphosphate (**II** in Fig. 1) and 2-aminopurine riboside 5′-triphosphate (**III** in Fig. 1) also produce fluorescent nucleotides that have been used to study the myosin ATPase mechanism.[5] However, many of the rate constants of the nucleotide–protein interaction are altered by such modifications of the purine ring. The situation with guanine nucleotide-binding proteins is even worse because virtually any modification of the purine ring of the guanine nucleotide results in loss of binding activity.[6]

Modifications that do not alter the purine or pyrimidine ring systems offer an alternative approach to fluorescent nucleotide analogs. For example, an ATP analog with an altered phosphoryl structure, namely adenosine-5′-triphosphoro-γ-1-(5-sulfonic acid)naphthylamine [(γ-AmNS)ATP (**IV**) in Fig. 1], which was a good substrate for *Escherichia coli* RNA polymerase, has been described.[7]

Modification of the ribose moiety of nucleotides offers, in most cases, a satisfactory approach in that the resultant analogs mimic more closely the behavior of their parent nucleotides than do pyrimidine or purine

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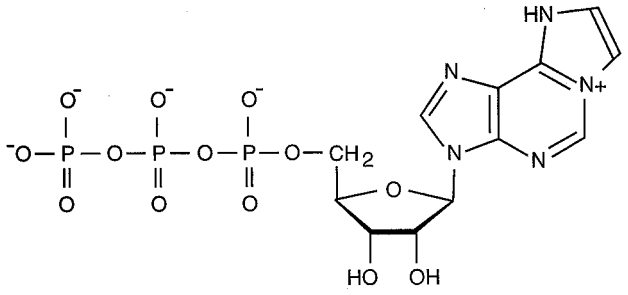
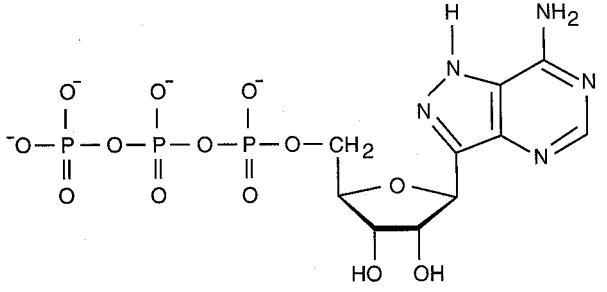


I



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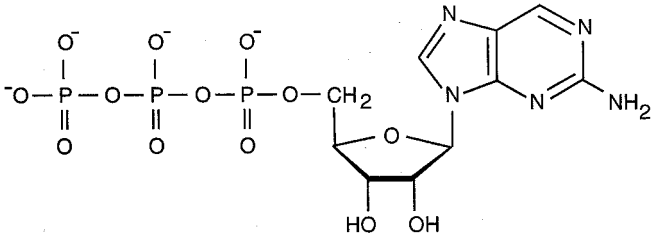

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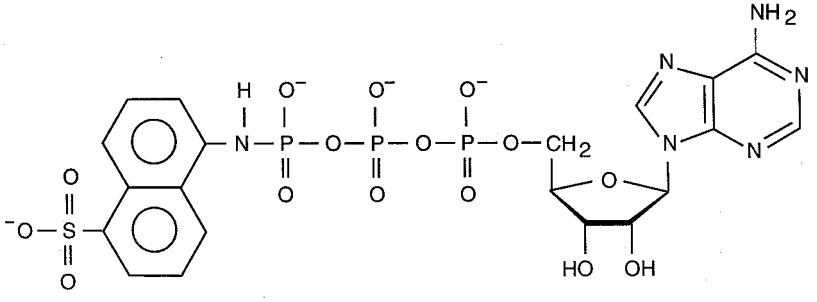
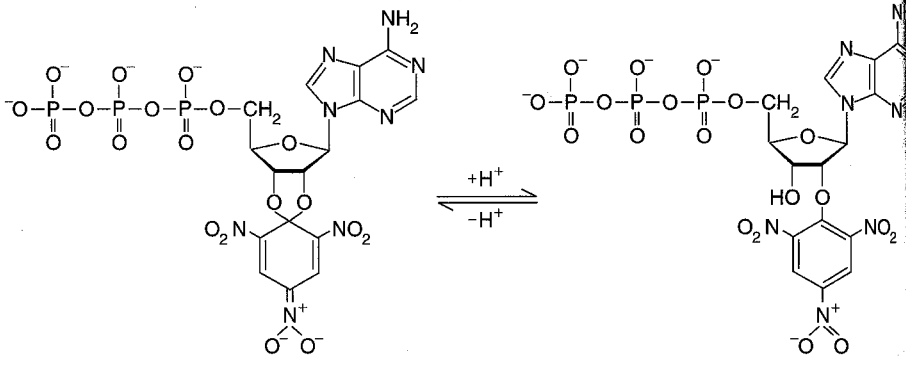


IV

FIG. 1. The structures of etheno-ATP **(I),** formycin 5′-triphosphate **(II),** 2-aminopurine ribose 5′-triphosphate **(III),** and (γ-AmNS)ATP **(IV).**



V

FIG. 2. TNP-ATP **(V)** exists as an equilibrium mixture depending on the pH. The right-hand structure represents the TNP group attached to either the 2′ or 3′ ribose hydroxyl.

riboside-modified analogs. We shall focus our attention on these derivatives. The benign nature of such ribose modification can be understood by examination of the structures of nucleoside 5′-triphosphatases such as myosin subfragment 1,[8] elongation factor Tu,[9–11] and $p21^{ras}$,[12] in which cases the 2′,3′-hydroxyl groups of the nucleotide project out of the binding domain. Scheidig *et al.*[13] determined the crystallographic structure of the catalytic domain of $p21^{H\text{-}ras}$ complexed with the nonhydrolyzable fluorescent analog, mantdGppNHp (mant probes are discussed in detail as follows) and found that the mant moiety was located on the surface of the protein.

The first fluorescent ribose-modified nucleotide appears to have been 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene) adenosine 5′-triphosphate (TNP-ATP) introduced by Hiratsuka and Uchida,[14] which exists as an equilibrium mixture (**V** in Fig. 2). Hiratsuka[15] then introduced the 2′(3′)-*O*-anthraniloyl (ant) and 2′(3′)-*O*-methylanthraniloyl (mant) derivatives of

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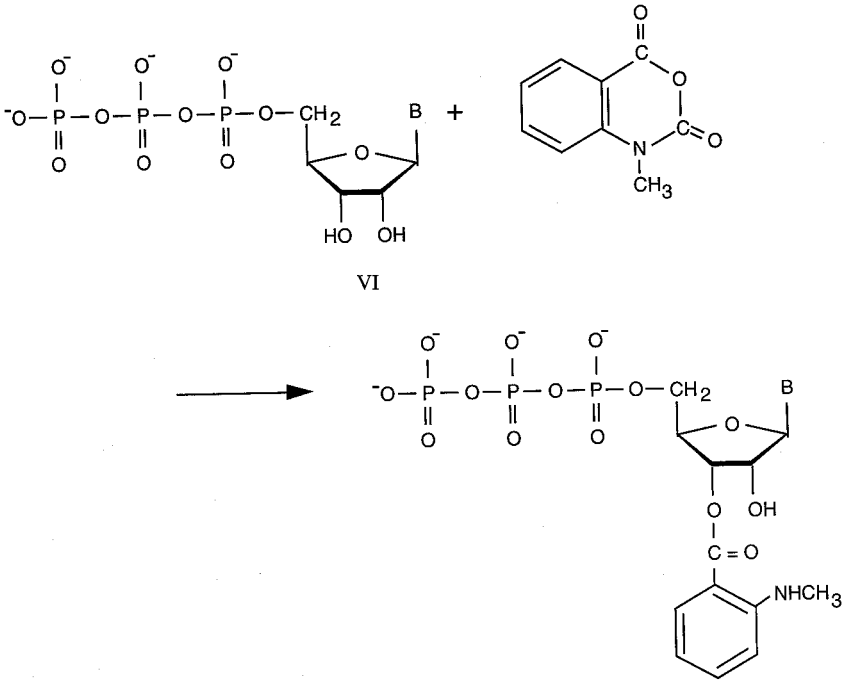

FIG. 3. The synthesis of mant-nucleotides **(VII)** by reaction of nucleotides with methylisatoic anhydride.

adenine and guanine nucleotides (**VII** in Fig. 3). One of the motivations for this choice of fluorophores was their relatively small size, which suggested that perturbation of binding properties would be minimal, and the fact that the fluorescence properties of these probes are environmentally sensitive. These probes have been used extensively in studies of nucleoside 5′-triphosphatases in a wide range of systems and closely mimic the parent nucleotides as regards rate and equilibrium constants, although there are exceptions. However, a more stringent test of their suitability is their ability to promote a particular biological process rather than interaction with a single protein. This concept is shown in Fig. 4, where the effects of ATP, εATP, and mantATP on the mechanical properties of permeabilized muscle fibers are shown. The ability of εATP and mantATP to produce tension in single muscle fibers (in the presence of calcium) and to relax them (in the absence of calcium) was compared to ATP. It can be seen that the εATP resulted in only 78% of the tension obtained with ATP whereas

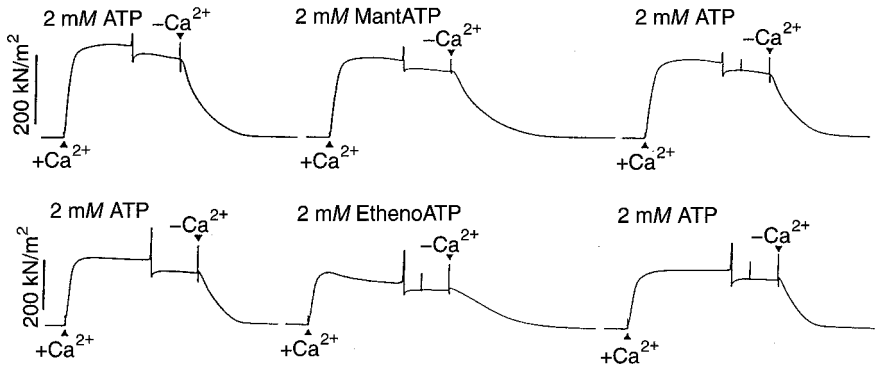

FIG. 4. A single permeabilized muscle fiber was incubated in the presence of ATP and Ca^{2+}, then ATP in the absence of Ca^{2+}, then either in the presence of EATP or mantATP in the presence and then absence of Ca^{2+}. The ATP cycle was then repeated. Muscle fiber tension was recorded with time. [Reprinted with permission from Woodward[71].]

mantATP gave a 95% increase in tension. The halftime of the relaxation process with εATP was 87% slower than with ATP but only 32% slower with mantATP. Similarly, Sowerby *et al.*[16] reported that mantATP supported movement of phalloidin-labeled actin filaments on immobilized rabbit skeletal muscle. On the other hand, Lark and Omoto,[17] studying axonemal dynein ATPases, showed that ant- and mantATP did not support the movement of wild-type *Chlamydomonas* axonemes, although they did reactivate sea urchin sperm axonemes.

Since the introduction of mant-nucleotides, other syntheses of fluorescent nucleotide analogs have been developed that allow a wide range of fluorophores to be introduced onto the ribose moiety. Jeng and Guillory[18] used the imidazolidate of carboxylic acids to acylate the 2′(3′)-hydroxyl of nucleotides (**IX** in Fig. 5) to produce photoaffinity labels and this approach has been extended to fluorescent analogs.[19,20] Cremo *et al.*[21] introduced a new class of analogs in which the fluorophore was attached to the ribose oxygens by a carbamoyl linkage. Hileman *et al.*[22] described the synthesis of fluorescein- and rhodamine-labeled GDP and ADP derivatives in which

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