Utility of a Novel Spin-Labeled Nucleotide in Investigation of the Substrate and Effector Sites of Phosphoribulokinase†

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ABSTRACT: The activated spin-label 3-(2-bromoacetamido)proxyl modifies the sulfur atom of phosphorothioate-containing AMP, ADP, and ATP analogs in a facile reaction that produces a new series of spin-labeled nucleotides. One of these products, adenosine 5′-*O*-(*S*-acetamidoproxyl 3-thiotriphosphate) (ATPγSAP), has been evaluated as a structural probe for *Rhodobacter sphaeroides* phosphoribulokinase (PRK). When incubated with affinity-purified enzyme that contains tightly bound substrate ATP, ATPγSAP binds noncooperatively to the allosteric site ($n = 1$; $K_D = 8$ μM). Probe bound in this site is displaced ($K_{1/2} = 100$ μM) by the allosteric effector, NADH, at concentrations comparable to those required for enzyme activation ($K_a = 133$ μM). In the presence of NADH, when PRK's substrate site is vacant, ATPγSAP binds in a cooperative mode (Hill coefficient ≈ 2.9; $K_D = 20$ μM). In the absence of NADH, ATPγSAP mimics ATP by exhibiting nonequilibrium binding to PRK. The observations with phosphoribulokinase, together with the straightforward nature of the methodology documented for synthesis and isolation of this class of spin-labeled nucleotides, suggest that these analogs have potentially wide application as structural probes.

Paramagnetic cofactors and substrate analogs have proven to be useful tools in our evaluation of the structural integrity of a variety of engineered enzymes (Misra et al., 1993; Narasimhan et al., 1995; Roberts et al., 1995). Recently, mutagenesis work on the active site of phosphoribulokinase (PRK[1]) (Sandbaken et al., 1992; Charlier et al., 1994), which catalyzes the regulated formation of ribulose 1,5-bisphosphate, the Calvin cycle's CO_2 acceptor, stimulated our interest in structural characterization of this enzyme. A logical extension of our use of paramagnetic probes involves identification of an appropriate spin-labeled analog that would occupy PRK's adenine nucleotide substrate site. Derivatization of the ATP's purine or ribose moieties has led to several spin-labeled nucleotide analogs (Ubom et al., 1989; Streckenbach et al., 1980; Alessi et al., 1992). However, a probe in which the paramagnetic moiety is linked to the phosphate chain of the nucleotide seemed potentially more useful for the study of PRK, since we had demonstrated (Miziorko et al., 1990) that derivatization of the nucleotide's phosphoryl chain did not preclude effective binding of a nucleotide analog to PRK.

The work of Connolly and Eckstein (1982) indicated that the sulfur atom of phosphorothioate-containing adenine nucleotides reacts selectively with alkylating agents. That precedent prompted our test of a potentially straightforward synthetic approach to produce spin-labeled phosphorothioate analogs of AMP, ADP, and ATP. This report describes the efficacy of that strategy and also demonstrates the utility of the spin-labeled ATP analog in investigation of the nucleotide substrate and dinucleotide effector sites of PRK. A preliminary account of this work has appeared (Koteiche et al., 1995).

MATERIALS

ATPγS, ADPβS, and 3-(2-bromoacetamido)proxyl were purchased from Sigma Chemical Co. AMP(S) was synthesized according to the procedure of Murray and Atkinson (1968) and purified by anion exchange chromatography. Ampicillin and isopropyl β-D-galactoside (IPTG) were purchased from United States Biochemical Corp. Reactive green-19 agarose and all other biochemicals and enzymes were obtained from Sigma Chemical Co. All other chemicals were of reagent grade and were used without further purification.

METHODS

Synthesis of Adenosine 5′-O-(S-Acetamidoproxyl thiomonophosphate) (AMPSAP) and Adenosine 5′-O-(S-Acetamidoproxyl 2-thiodiphosphate) (ADPβSAP). AMP(S) (30 μmol) was dissolved in methanol, and the pH was adjusted to ~4.5. This solution was brought to a 1.5-fold excess of 3-(2-bromoacetamido)proxyl; the final reaction

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[1] Abbreviations: PRK, phosphoribulokinase; Ru5P, ribulose 5-phosphate; AMP(S), adenosine 5′-*O*-(thiophosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); proxyl, 2,2,5,5-tetramethyl-1-pyrrolidinyloxy; tempo, 2,2,6,6-tetramethyl-1-piperidinyloxy; AMPSAP, adenosine 5′-*O*-(*S*-acetamidoproxyl thiophosphate); ADPβSAP, adenosine 5′-*O*-(*S*-acetoamidoproxyl 2-thiodiphosphate); ATPγSAP, adenosine 5′-*O*-(*S*-acetoamidoproxyl 3-thiotriphosphate); ADP-R•, adenosine 5′-diphosphate-4-(2,2,6,6-tetramethylpiperidine-1-oxyl); TEA, triethylammonium; ESR, electron spin resonance.

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volume was 1 mL. The reaction was allowed to proceed at room temperature and was complete within 10 min, as indicated by ^{31}P NMR (chemical shift of derivatized phosphorothioate: 20.26 ppm). The product AMPSAP was recovered by precipitation from cold (0 °C) ethyl ether. Similar conditions were employed to form ADPβSAP. Commercially available adenosine 5′-*O*-(2-thiodiphosphate), which ^{31}P NMR showed to be acceptable for use without prior purification, was used as starting material. Upon derivatization of ADPβS, chemical shift of the β-phosphorothioate doublet (measured in deuterated methanol) changes from 40.2 to 9.5 ppm. The ether-precipitated products were dried under nitrogen. Stock solutions, prepared by dissolving the precipitated nucleotides in methanol, were stored at −20 °C.

Synthesis of Adenosine 5′-O-(S-Acetamidoproxyl 3-thiotriphosphate) (ATPγSAP). Commercially available (Sigma) adenosine 5′-*O*-(3-thiotriphosphate) was shown by ^{31}P NMR to contain ADP as an ~20% contaminant; this contaminant is not easily resolved from the spin-labeled product and, thus, must be eliminated by chromatographic purification of the starting material. ATPγS (13 mg) was dissolved in 10 mL of TEA bicarbonate and loaded on a 1.5 × 20 cm DEAE-Sephadex column equilibrated with 200 mM TEA bicarbonate. The column was then washed with two column volumes of equilibration buffer and eluted with a 500 mL gradient (200 to 700 mM TEA bicarbonate). The nucleotide-containing fractions were monitored at 260 nm, and those corresponding to the last peak of the absorption profile were pooled and desalted by five to six evaporations from anhydrous methanol. After conversion of an aqueous solution of the nucleotide to the free acid using Dowex 50 (H^+ form), the pH of the sample was adjusted from 1.5 to 5.0 using lithium hydroxide. ^{31}P NMR (Jaffe & Cohn, 1978) verified the purity of the ATPγS. For derivatization, a solution of ATPγS (~12 μmol) was prepared in 1 mL of anhydrous methanol and added to a 1 mL solution containing 22 μmol of 3-(2-bromoacetamido)proxyl in methanol. The reaction was allowed to proceed at room temperature for 40 min and was complete when checked by ^{31}P NMR. Chemical shift of the doublet assigned to the γ-phosphorothioate changes from 41.0 to 10.3 ppm upon derivatization. The product ATPγSAP was recovered by ether precipitation, dried under nitrogen, and stored at −20 °C.

Expression, Purification, and Assay of PRK. Escherichia coli BL21 cultures containing plasmid pETbprkwt (Charlier et al., 1994) were grown in ampicillin-containing LB media to a final $OD_{600} \sim 0.7$. Expression of PRK was induced by the addition of IPTG to a final concentration of 1 mM. The cells were grown for an additional 3−4 h at 25 °C. Cell pellets were prepared and disrupted in a French pressure cell. Except where indicated, enzyme was isolated using the Q-Sepharose anion exchange chromatography and reactive green-19 agarose affinity chromatography protocols of Charlier et al. (1994). From 1.6 L of induced bacterial culture, an average of 24 mg of pure enzyme was recovered by the above method.

A modified version of the above procedure was used to isolate protein that contains no bound ATP. The procedure involved elution of PRK from reactive green-19 agarose affinity resin with 25 mM Tris-HCl (pH 8.2) containing 10 mM β-mercaptoethanol, 1 mM EDTA, and 1 M KCl. This step was followed by gel filtration chromatography using Superose 6 resin to yield a protein of specific activity comparable to standard preparations.

Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin used as standard. PRK activity was measured by the spectrophotometric method of Krieger and Miziorko (1986) or by the radioactive assay described by Charlier et al. (1994).

Measurement of the NADH and ATP content of purified PRK samples was performed by fluorescence measurements. The NADH content of enzyme samples was determined by measuring the 440 nm fluorescence (excitation at 340 nm) and comparison of observed values with a calibration curve constructed using appropriate NADH concentrations (Ehrig et al., 1994). The ATP content was determined by the hexokinase reaction in an assay (Van der Kuil & Korf, 1991) that couples ATP dependent glucose-6-phosphate formation to the production by glucose-6-phosphate dehydrogenase of NADPH, which can be sensitively estimated by fluorescence measurements (emission wavelength, 440 nm; excitation wavelength, 340 nm).

Nuclear Magnetic Resonance (NMR) Measurements. ^{31}P NMR spectra of methanolic solutions of the spin-labeled nucleotides were recorded at 121.5 MHz on a Bruker AC-300 spectrometer equipped with a multinuclear probe (5 mm) and quadrature phase detection. The field was locked on deuterium, and all spectra were recorded with broad-band proton decoupling. Spectra were recorded at 23 ± 1 °C.

Electron Spin Resonance (ESR) Measurements. A Varian E-109 X-band spectrometer equipped with a TE-102 cavity was used for ESR measurements. Samples were loaded in 20 μL capillaries, sealed at one end, and positioned within the cavity inside a 4 mm quartz tube. Spectra were recorded at the following instrument settings: microwave frequency, 9.506 GHz; microwave power, 5 mW; modulation amplitude, 1 G; modulation frequency, 100 KHz; time constant, 0.128 s; and scan width, 100 G. All the spectra were recorded at room temperature (22 °C). All samples were prepared in 25 mM Tris-HCl (pH 8.2) containing 5 mM $MgCl_2$. The binding of spin-label to PRK was monitored by comparison of the peak-to-peak amplitude of the high-field line of a sample containing ATPγSAP and enzyme with the amplitude observed with a solution containing an equal concentration of ATPγSAP in buffer.

Scatchard Analysis. The concentrations of bound and free ATPγSAP were determined from the ESR spectra as indicated above. The data plots were fitted by linear regression analysis to the equation

$$B/F = (-1/K_D)B + n/K_D$$

B is bound ATPγSAP normalized per enzyme site, F is free ATPγSAP, K_D is the dissociation constant, and n is the number of sites, as determined from the x-intercept of the data plot. In the case of linear independent binding, the K_D was determined from the slope or the y-intercept. In the cooperative mode of binding, the data were linearized (Schreier & Schimmel, 1974) using the equation

$$\ln[F] = (-1/a)\ln[(n/B) - 1] + \ln K_D$$

in which a is the Hill coefficient and n is the number of sites determined from the extrapolated x-intercept of the Scatchard plot.

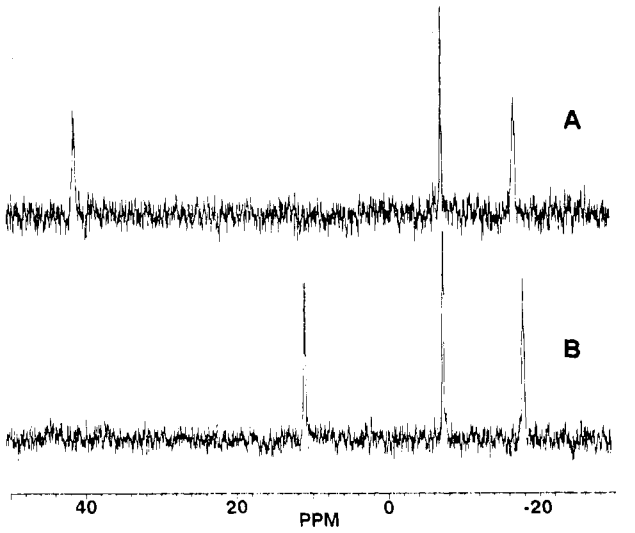
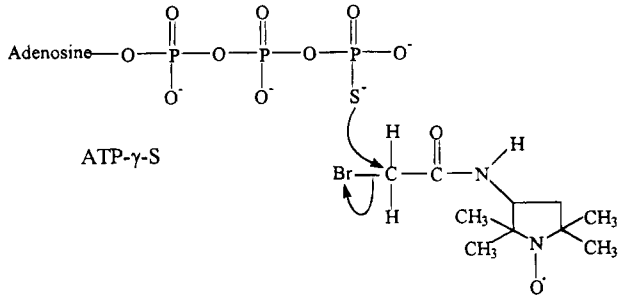


FIGURE 1: (A) ^{31}P NMR spectrum (1000 scans) of ATPγS (1.2 mM) purified by ion exchange chromatography. (B) ^{31}P NMR spectrum (134 scans) of ATPγS coupled to 3-(2-bromoacetamido)-proxyl (6.0 mM). The γ-phosphate peak shifts from 41 to 10.5 ppm after the reaction is completed.

Scheme 1



RESULTS

Synthesis and Characterization of Adenosine 5′-O-(S-Acetamidoproxyl thiomono-, di-, and triphosphates). Reaction of 3-(2-bromoacetamido)proxyl (1.5−1.8-fold molar excess over nucleotide) with methanolic solutions of AMPS, ADPβS, or ATPγS converts each nucleotide to the respective *S*-acetamidoproxyl derivative. ^{31}P NMR indicates that the reactions proceed rapidly (<40 min) to completion. For example, in the case of ATPγS (Scheme 1), derivatization changes the chemical shift of the doublet resonance attributable to the γ-phosphorothioate from 41 to 10 ppm (Figure 1). Ether precipitation of the lithium salt of the derivatized nucleotides results in the recovery of the respective products in yields of 50−70%. The isolated nucleotides are free of any trace of the modification reagent, which is required in only slight excess. Double integration of the ESR signal (Hyde et al., 1992) of aqueous solutions of AMPSAP, ADPβSAP, or ATPγSAP (Figure 2) indicates that proxyl free radical spin levels are stoichiometric with respect to A_{260} estimates of adenine nucleotide concentration. The 9 GHz ESR spectrum (Figure 2) displays reduced intensity of the high-field peak due to ATPγSAP in comparison with that observed for spin-label that is not covalently linked to nucleotide ($\tau_c = 4 \times 10^{-11}$ s), indicating the expected motional constraint ($\tau_c = 2 \times 10^{-10}$ s).

Binding of ATPγSAP to the Allosteric Site of Rhodobacter sphaeroides Phosphoribulokinase. On the basis of our previous observations (Miziorko et al., 1990) that PRK binds the affinity label adenosine *tri*phosphopyridoxal >15-fold more tightly than the corresponding *di*phosphopyridoxal analog, ATPγSAP was selected for detailed investigation as a structural probe for PRK. The recombinant *R. sphaeroides* enzyme, an octamer of 32 kDa subunits, is isolated in homogeneous form by ATP elution of the enzyme from a reactive green-19 agarose affinity column (Sandbaken et al., 1992; Charlier et al., 1994). Purified enzyme is subjected to centrifugal gel filtration or extensive dialysis prior to concentration for ESR measurements. Binding of ATPγSAP to PRK prepared under such conditions is easily detected upon inspection of the ESR spectrum of this sample; the sharp peaks attributable to free analog are markedly diminished in amplitude, and the broad features of PRK-bound analog become apparent (Figure 2). The spectral line shape indicates considerable motional freedom ($\tau_c = 3.2$ ns) in

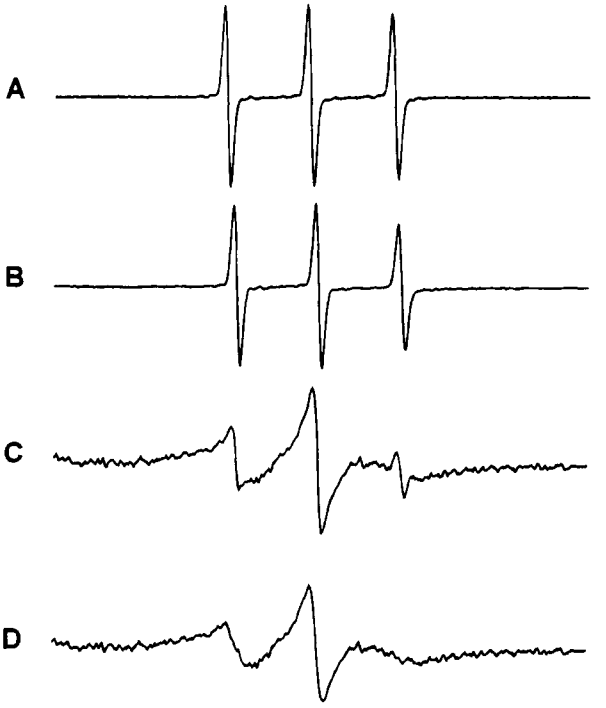


FIGURE 2: X-band ESR first derivative spectra of free and enzyme-bound ATPγSAP. (A) ESR spectrum of free 3-(2-bromoacetamido)-proxyl. The concentration of the sample was 100 μM. The instrument settings are as follows: microwave frequency, 9.506 GHz; microwave power, 5 mW; modulation amplitude, 1 G; modulation frequency, 100 KHz; time constant, 0.128 s; gain, 5×10^3; and scan range, 100 G. Instrument settings are the same for all spectra below unless otherwise noted. (B) ESR spectrum of free ATPγSAP (24 μM); instrument gain = 2×10^4. (C) ESR spectrum of ATPγSAP (24 μM) bound to PRK (450 μM site concentration). Instrument gain is 4-fold higher (8×10^4) than that used for spectrum B. Additionally, due to normalization of spectral output by the data analysis program (Hyde et al., 1992), spectrum C is further amplified by 1.4-fold relative to spectrum B. Two components with different rotational mobilities are apparent. The bound ATPγSAP component ($\tau_c = 3.2$ ns; Kivelson, 1960) exhibits a broad spectral feature underlying the signal attributable to free ATPγSAP ($\tau_c = 0.2$ ns). The amount of free and bound probe is determined by comparison of the amplitude of the high-field resonance line of the ATPγSAP spectrum (normalized for any differences in instrumental gain or amplification) in the presence and absence of PRK. (D) Difference spectrum in which 5% of the total signal (spectrum B) of the aqueous solution of ATPγSAP (normalized to the spectral conditions used for spectrum C) is subtracted from the spectrum of PRK-bound ATPγSAP (spectrum C), giving the spectrum attributable to the bound component.

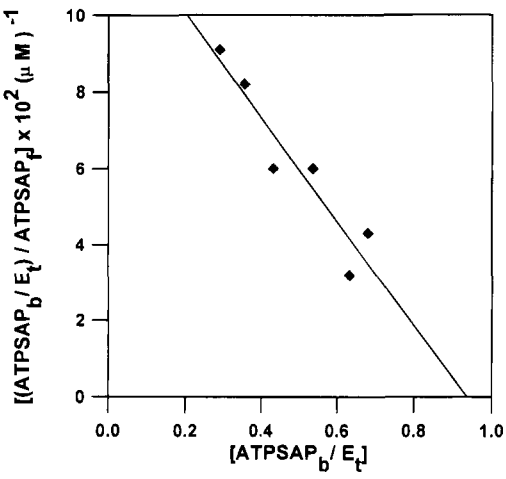


FIGURE 3: Analysis of PRK·ATPγSAP equilibrium binding data. The samples were prepared such that the concentration of the probe was kept constant at 21 μM and the protein concentration was varied between 5 and 69 μM in 25 mM Tris-HCl buffer (pH 8.2). Enzymatic assay of PRK used in these experiments indicates that 1 ATP molecule is bound per 32 kDa PRK monomer. The concentrations of free and bound probe were determined from the ESR spectra as described under Methods. Linear regression analysis of the data indicate a $K_D = 8$ μM and binding stoichiometry, $n \approx 1$. These estimates are in good agreement with values ($K_D = 7$ μM; $n = 0.9$) obtained by a theoretical fit of the data to a binding isotherm.

comparison with the rotational dynamics ($\tau_c \approx 76$ ns) expected for a 256 kDa octameric ensemble of PRK subunits. PRK exhibits stoichiometric, high-affinity ($K_D = 8$ μM), noncooperative binding of the spin-labeled ATP analog, as demonstrated by Scatchard analysis of the data (Figure 3). Since *kinetic* characterization of the prokaryotic PRKs (Sandbaken et al., 1992; Rippel & Bowien, 1984) suggested that, under assay conditions, ATP binds with positive cooperativity, the observed noncooperative formation of a binary PRK·ATPγSAP complex raised an issue concerning the identity of the site occupied by the probe. Prokaryotic PRKs bind the *mono*nucleotide ATP at the catalytic site and the *di*nucleotide NADH (a positive effector) at an allosteric site. Comparison of the structure of ATPγSAP with those of ATP and NADH (Figure 4) indicates that the analog may mimic the ADP-ribose portion of NADH, allowing it to bind effectively to PRK's allosteric site. This hypothesis can be tested by measuring the ability of NADH to displace probe from the complex with PRK. Results of such an experiment (Figure 5) are consistent with the binding site assignment; NADH does, in fact, displace the probe. The displacement occurs at concentrations ($K_{1/2} = 100$ μM) comparable to those required for NADH stimulation of PRK activity. The kinetic data (Figure 6) indicate hyperbolic (noncooperative) saturation of enzyme by this effector ($K_a = 133 \pm 24$ μM).

Observation of allosteric site occupancy by the stoichiometrically bound spin-labeled ATP analog raised an issue concerning accessibility to the substrate binding site. ATP analysis of protein preparations used for the experiments outlined above indicated that PRK prepared by ATP elution of affinity columns contains stoichiometric levels of ATP, even after centrifugal gel filtration or extensive dialysis. The presence of such prebound ATP would account for occupancy of the effector site by ATPγSAP.

Binding of ATPγSAP to the Substrate Site of PRK. To test whether the spin-labeled analog could bind at the substrate site, the standard PRK preparation, which contains tightly bound ATP, was incubated with activator NADH, divalent cation, and cosubstrate Ru5P under catalytic turnover conditions. ATP analysis verified displacement of the nucleotide substrate by this protocol. The protein was subsequently dialyzed and concentrated for use in an ESR evaluation of ATPγSAP binding. Scatchard analysis of the data (Figure 7A) indicates positive cooperativity in binding to vacant substrate site. Linearization of the binding data (Schreier & Schimmel, 1974; Figure 7B) indicates a Hill coefficient ≈ 2.9 and $K_D = 20$ μM. Under steady state kinetic conditions, NADH is required for cooperativity in substrate ATP binding (J. Runquist, unpublished observations). In accordance with this observation, analysis for NADH in the PRK preparation, isolated after ATP turnover and used for this ESR binding experiment, indicated that stoichiometric levels of bound NADH persisted through the dialysis and concentration steps used in preparing protein for the ESR measurements. In support of these results, after methodology to isolate ATP-free PRK was developed (*vide infra*), preincubation of enzyme with NADH prior to evaluation of ATPγSAP binding resulted in observations comparable to those shown in Figure 7. The combined data demonstrate the requirement for NADH to support cooperative binding at the ATP site.

Nonequilibrium Binding of ATPγSAP to PRK. In contrast to the equilibrium binding exhibited by ATPγSAP in occupying the NADH site when ATP is prebound and the substrate ATP site when NADH is present, ATP exhibits *non*equilibrium binding to PRK after affinity chromatography. It might be anticipated that ATPγSAP would exhibit similar nonequilibrium binding under certain experimental conditions. PRK can be isolated at a high level of purity by a modified procedure involving KCl elution of reactive green-19 agarose affinity resin followed by gel permeation chromatography using Superose 6 resin. Enzyme isolated by this modified approach contains no bound ATP when evaluated by a hexokinase-based assay but will, upon incubation with excess ATP, bind stoichiometric levels of the nucleotide, even after prolonged dialysis. Measurements of ATPγSAP bound to ATP-free PRK preparations show that the available enzyme sites are titrated by the analog in this nonequilibrium binding mode (Figure 8). In subsequent experiments on PRK that is occupied by the analog in this binding mode, the anticipated displacement of prebound ATPγSAP by ATP was demonstrated; not unexpectedly, the kinetics of displacement are quite slow (end points after ≈1 h).

DISCUSSION

Given the large number of proteins that bind or metabolize adenine nucleotides, synthesis of nucleotide analogs into which reporter groups have been incorporated has been a pursuit attractive to a variety of investigators. In the context of spin-labeled analogs, early work on derivatization of the ribose ring of ATP (Streckenbach et al., 1980) represented a prelude to production of nucleotides containing both photoaffinity and spin-label probes (Vogel et al., 1988; Jacobs et al., 1989). Subsequently, synthesis of an analog in which the spin-label is attached to ribose as a spiroketal was reported to restrict conformational mobility (Alessi et al., 1992). Additionally, spin-labeled adenine nucleotide

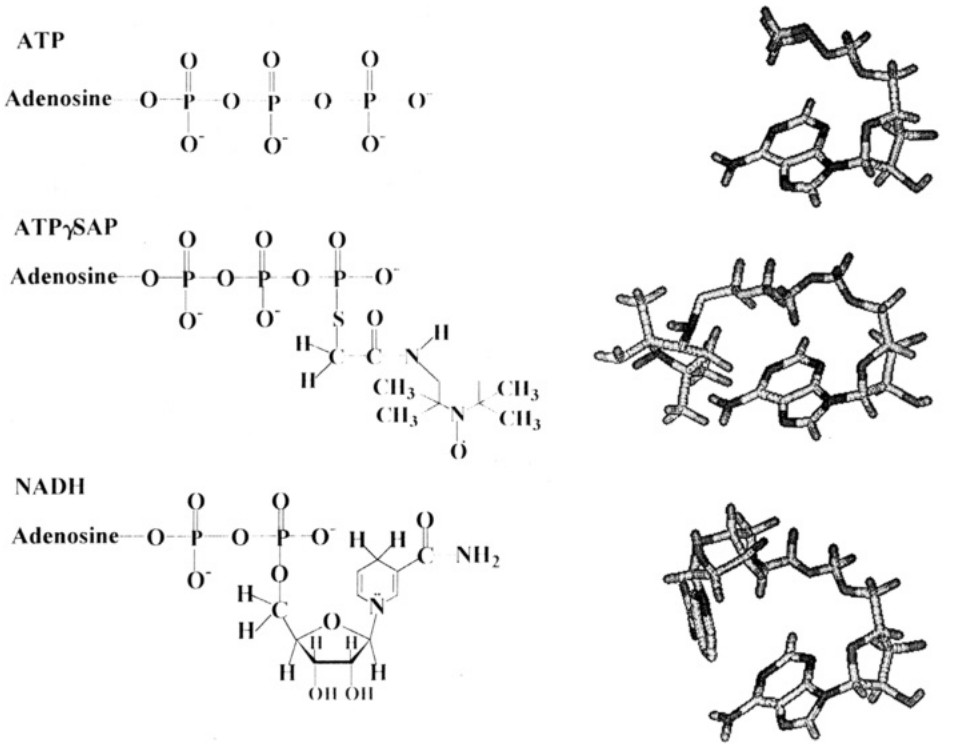


FIGURE 4: Comparison of the structures of the PRK substrate, the spin-labeled nucleotide, and a PRK allosteric effector. The right side of the figure depicts "cylinder" representations of the molecules in the energy-minimized conformations predicted by the Alchemy modeling program.

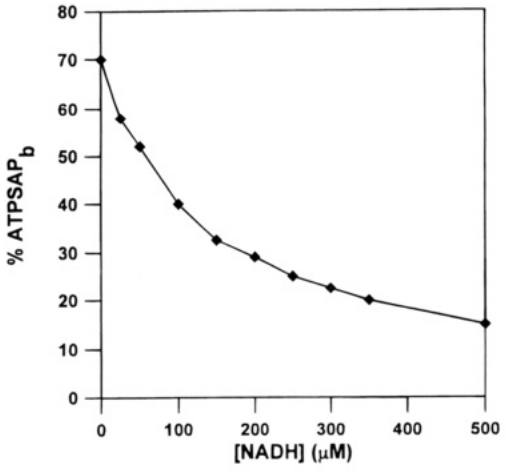


FIGURE 5: Titration curve showing the displacement of ATPγSAP by NADH. The concentrations of PRK and ATPγSAP were kept constant at 37 and 23 μM, respectively; samples were prepared with different concentrations of NADH, as indicated. $K_{1/2}$ determined from the displacement experiment was 100 μM.

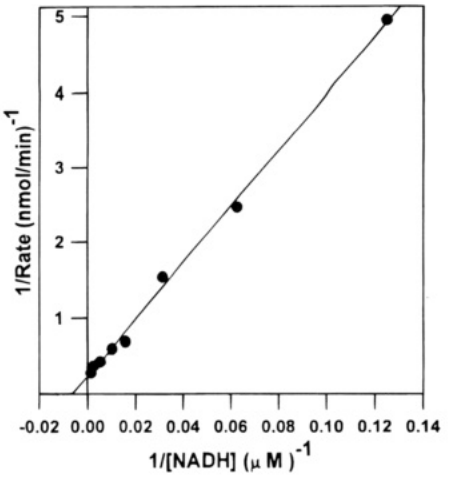


FIGURE 6: Allosteric activation of PRK by NADH. Activity, determined by the radioactive assay (cf. Methods), was measured at 30 °C in reaction mixtures containing 0.11 μg of PRK and NADH ranging from 8 to 800 μM. A double reciprocal plot of the data is shown. K_a = 133 μM was determined by nonlinear regression analysis of the data.

analogs in which the probe is linked to purine C6 have been reported (Ubom et al., 1989).

While analogs of the classes listed above have proven useful and, in many cases, function as substrates for the enzymes under investigation, it also seems attractive to position a reporter group on the nucleotide's terminal phosphoryl group, the site where catalysis frequently occurs. While such an analog is not likely to function as a substrate, steric hindrance did not appear, *a priori*, to pose a serious concern. Stereochemical precedent for inversion of configuration during phosphoryl transfer (Eckstein, 1983; Knowles, 1980) argues that many enzymes simultaneously accommodate both phosphoryl acceptor and adenine nucleotide phosphoryl donor. Thus, spin-label attached to the terminal phosphoryl moiety of a nucleotide should, in most cases, not hinder binding. In the particular case of PRK, our previous affinity-labeling work involved derivatization of the phosphate chain with a pyridoxal group to produce a tight binding (K_d = 11 μM) analog (Miziorko et al., 1990). This result argued that similar attachment of a proxyl moiety could lead to a productive probe; the ATPγSAP binding data presented above validate this prediction.

Formation of a phosphodiester linkage between tempo phosphate and AMP produces the ADP-R• analog of Weiner (1969), which was utilized to study the dinucleotide site on alcohol dehydrogenase. Synthesis and isolation of ADP-R•, which may be viewed as a model for the type of spin-

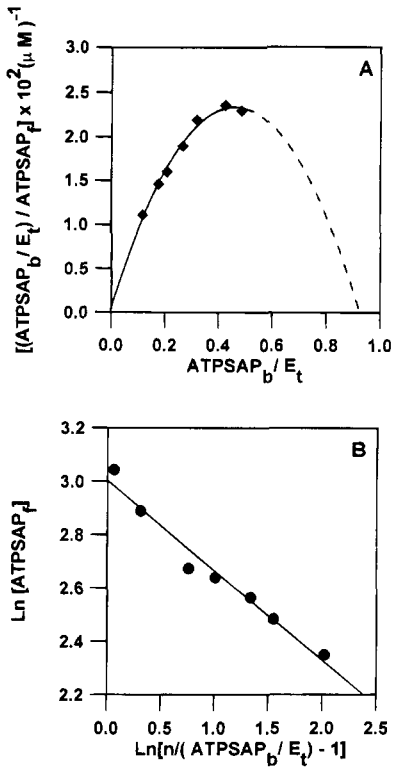


FIGURE 7: Cooperative binding of ATPγSAP to PRK. (A) Scatchard plot of ESR data for ATPγSAP binding to PRK. Metabolite analyses indicated that the PRK preparation contained 1 NADH and <0.2 ATP molecule per 32 kDa monomer. For sample preparation, the ATPγSAP concentration was kept constant at 25 μM and PRK site concentration was varied from 8 to 124 μM. The data were fit to a second degree polynomial function, generating a curve with an x-axis intercept that approaches unity. (B) The data from panel A were linearized using the equation $\ln[F] = -1/a \ln[n/B-1] + \ln K_D$, where a is the Hill coefficient. Using a value for $n \sim 1$, as suggested by the theoretical fit to the data shown in panel A, estimates of $a \sim 2.9$ and $K_D \sim 20$ μM were calculated.

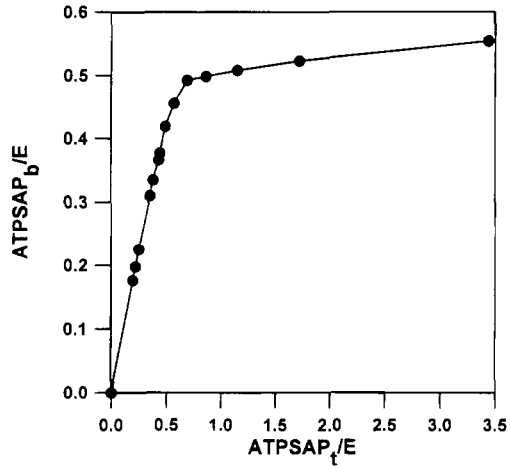


FIGURE 8: Titration curve showing nonequilibrium binding of ATPγSAP to PRK. Protein for this experiment was purified by a modified protocol involving KCl elution of affinity resin. This PRK preparation was analyzed by metabolite assays and found to contain no tightly bound ATP. Samples contained 21 μM ATPγSAP; PRK protein concentration varied from 6 to 112 μM.

labeled analog on which we now report, requires activation of both phosphorylated spin-label and nucleotide prior to phosphodiester formation as well as chromatographic purification of the reaction product. The synthesis of our phosphorothioate-based analogs, which is based on the seminal work of Connolly and Eckstein (1982), can be rapidly accomplished and involves considerably less effort. Moreover, the protocol affords product in reasonable yield, even working at a micromole scale. The attractiveness of the phosphorothioate-based derivatization strategy is also apparent from recent work in which reactive affinity labels were developed (Vollmer et al., 1994).

In evaluating the utility of ATPγSAP as a structural probe of PRK, we demonstrate that it can occupy both substrate ATP and activator NADH sites. In occupying the substrate ATP site in the presence of NADH, the analog binds with positive cooperativity, as observed for ATP occupancy of PRK (Hill coefficients of 1.7−2.6) from a variety of organisms (Hart & Gibson, 1971; MacElroy et al., 1972; Rippel & Bowien, 1984). $K_{1/2}$ values for ATP ($\sim 5 \times 10^{-4}$ M), as reported in steady state kinetic experiments, are typically higher than the K_D for the spin-labeled ATP analog. However those $K_{1/2}$ values, measured in the presence of both substrates and effector, are not directly comparable to K_D for the E·ATPγSAP complex, measured in the absence of Ru5P in our physical-binding experiments. In the context of the results of Weiner (1969) with ADP-R•, it was not unexpected that ATPγSAP can occupy PRK's dinucleotide site. In binding to this site, the analog does not support activation of the enzyme. This is not surprising, given that metabolites such as NAD^+ and NADPH, which are structurally more closely related to NADH, do not activate PRK (Rindt & Ohmann, 1969). Initially, the ability of the ATPγSAP to bind tightly to both catalytic and allosteric sites complicated analysis, but as methodology developed that allowed selective occupancy of either site, the utility of the analog has been enhanced. Key to directing probe binding was the discovery that PRK can exhibit nonequilibrium stoichiometric binding of ATP. This observation may be attributable to a preferred order of substrate binding, although such a possibility is speculative since direct studies addressing this issue have not been reported. We have, however, determined that ATP bound in the nonequilibrium mode will turn over to form the reaction product, ribulose 1,5-bisphosphate (H. Charlier, unpublished), indicating that the substrate site is occupied. In titrating the ATP-free enzyme with ATPγSAP (Figure 8), we demonstrate that analog will also bind in a nonequilibrium fashion, albeit at a stoichiometry (4−5 per octamer) less than observed when the same sample is titrated with ATP (8 ATP/octamer). This difference may be due to steric perturbation inherent in derivatization of the γ-phosphoryl of the analog or perhaps due to a conformational change in the PRK octamer that is prompted by binding of authentic substrate ATP but not by binding of the analog ATPγSAP, which contains structural features distinct from substrate (Figure 4). Discrimination between these or other explanations will require further investigation using different analogs or alternative physical techniques.

Whether in the context discussed above or in evaluation of the structural integrity of PRK mutants on the basis of ATPγSAP binding properties, this spin-labeled nucleotide shows considerable promise as a valuable structural probe. Extension of such work to other nucleotide binding proteins also promises to be productive and would enhance the significance of this series of analogs. A future survey of how discrimination between occupancy of mono- and dinucleotide sites on PRK or other proteins varies as a function of the number of phosphoryl moieties in the analog should

prove interesting. Preliminary experiments on PRK suggest that ADPβSAP will be useful in such studies.

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