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INTERACTION OF GLYCOGEN PHOSPHORYLASE WITH 8-AZIDOADENOSINE 5'-MONOPHOSPHATE, A PHOTOAFFINITY ANALOG OF AMP

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Summary

The ability of 8-azidoadenosine 5'-monophosphate (N_3AMP) to act as a photoaffinity label for the AMP binding site on glycogen phosphorylase (EC 2.4.1.1) was tested. 8-Azidoadenosine 5'-monophosphate can replace AMP as an allosteric modifier of both phosphorylases *a* and *b*; the pH optimum and the extent of activation are comparable to that observed with AMP. 8-Azidoadenosine 5'-monophosphate resembles the natural activator in having a higher affinity for phosphorylase *a*. The effects of 8-azidoadenosine 5'-monophosphate and AMP on phosphorylase *b* are additive when each is present at a concentration which gives less than 50% activation. Increasing the concentration of the substrate, glucose 1-phosphate, decreases the apparent activation constant (K_a) for the interaction of 8-azidoadenosine 5'-monophosphate with phosphorylase *b*. Glucose 6-phosphate is an inhibitor of phosphorylase *b* with either AMP or 8-azidoadenosine 5'-monophosphate. In the presence of ultraviolet light, 8-azidoadenosine 5'-monophosphate is irreversibly incorporated into phosphorylase *a*; incorporation at the allosteric site can be reduced if AMP is added prior to irradiation. Under the conditions used in the photolysis experiments, 3—5% of the available AMP sites were labeled with 8-azidoadenosine 5'-monophosphate. The data indicate the potential usefulness of 8-azidoadenosine 5'-monophosphate as a probe for the AMP site on phosphorylase.

Introduction

Glycogen phosphorylase (EC 2.4.1.1) is a regulatory enzyme whose activity is modulated by a complex mechanism which includes activation by AMP. The

Abbreviations: N_3AMP, 8-azidoadenosine 5'monophosphate; BrAMP, 8-bromoadenosine 5'-monophosphate; $\overline{n}$, average number of mol of N_3AMP bound/mol of the phosphorylase monomer.

enzyme from rabbit muscle exists in two interconvertible forms: phosphorylase *b*, a dimer inactive in the absence of AMP; and phosphorylase *a*, a tetramer which exhibits 70—85% of its maximum activity in the absence of the nucleotide. An understanding of the interaction between AMP and the amino acid residues forming the binding site may shed light on the mechanism of control at a molecular level. Recently, outstanding contributions have been made to our knowledge of the physical and chemical structures of the enzyme. The complete amino acid sequence of the phosphorylase monomer has been elucidated [1]. Models for the backbone conformation of the two polypeptides which comprise the dimers of phosphorylases *a* [2] and *b* [3] have been presented from X-ray diffraction data at 3 Å resolution. The position of the coenzyme, pyridoxal phosphate, has been located and the binding domains of several ligands including AMP, glucose 1-phosphate, glucose and maltoheptose have been outlined [4—6]. The site of attachment of an analog of AMP, 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine, was identified as tyrosine-155 in the primary sequence of phosphorylase [1]. Anderson et al. [7] postulated that the sulfonyl fluoride group of the derivative labels a residue which is normally in the vicinity of the phosphate group of enzyme-bound AMP. A more complete characterization of the allosteric site on phosphorylase requires the identification of the amino acid residues which interact with the purine ring of AMP.

An analog of AMP, 8-azidoadenosine 5′-monophosphate (N_3AMP), is a member of a class of compounds which resemble natural effectors but which are capable of generating a highly reactive intermediate. When activated by ultraviolet light, the compounds can form a covalent bond with any amino acid side chain in the vicinity of the binding site, permitting subsequent identification and analysis of the site [8]. The coenzyme sites which have been irreversibly labeled with 8-azidoadenine derivaties include the cyclic 3′,5′-AMP binding sites of erythrocyte membranes [9,10], the ADP binding site of glutamate dehydrogenase [11], the ATP binding site of mitochondrial ATPase [12], and the coenzyme A binding site on the enzyme, acyl-CoA:glycine *N*-acetyltransferase [13]. This report describes experiments which establish that N_3AMP can replace AMP as an allosteric modifier of phosphorylase and presents initial studies on the covalent incorporation of the analog into the protein.

Materials and Methods

Experimental procedure

Phosphorylase *b* was prepared from frozen rabbit muscle essentially according to the method of Fischer et al. [14] and recrystallized 3—4 times. Phosphorylase *a* was prepared from phosphorylase *b* using purified phosphorylase *b* kinase [15]. Nucleotides were removed from solutions of the enzyme either by treatment with acid-washed Norit or by gel filtration with Sephadex G-25. The ratio of absorbances (A_{260}/A_{280}) was less than 0.56 for all preparations of phosphorylases *a* and *b*. Enzymic assays were carried out in the direction of glycogen synthesis following the procedure of Hedrick and Fischer [16] except that 0.05 M sodium glycerophosphate (pH 6.8) was used instead of the maleate buffer. When assayed in the presence of AMP, the enzymes had

a specific activity of 90—100 μmol P_i from glucose-1-*P*/min per mg. The protein concentration was determined spectrophotometrically using an absorbance index at 280 nm (1%, 1 cm) of 13.2 [17]. Molar values were calculated using molecular weight 97 400 for the phosphorylase monomer [1].

Glucose-1-*P* (Sigma) was recrystallized from ethanol after precipitation of the contaminating inorganic phosphate with Ba^{2+}. Shellfish glycogen (Sigma) was purified by treatment of a 1% solution with Norit A followed by passage of the solution through a column of Dowex 1-X8(Cl^-). All other chemicals and biochemicals were obtained from commercial sources and were of the highest purity available. Deionized water was used to prepare all solutions.

BrAMP, N_3AMP, and [^{14}C]N_3AMP were prepared from AMP or uniformly labeled [^{14}C]AMP (New England Nuclear) by the method of Haley [9,18]. The purity of all preparations was assessed by several methods (vide infra) including thin-layer chromatography on cellulose sheets (Eastman 13254) using a solvent system containing 1-butanol/acetic acid/water (5 : 2 : 3, v/v). The chromatograms were developed with and without prior irradiation of the sample on the cellulose matrix with ultraviolet light (254 nm). The R_F values for N_3AMP and the principal photoproduct of N_3AMP are 0.50 and 0.38, respectively. The chromatograms of [^{14}C]N_3AMP were cut into sections and the radioactivity of each section was determined by liquid scintillation counting. Samples of non-radioactive N_3AMP were pure as judged by thin-layer chromatography and had an absorption maximum at 281 nm [19]; after irradiation in water, the spectrum was indistinguishable from that of other photolyzed 8-azidonucleotides [11,18]. The preparation of [^{14}C]N_3AMP contained a major component representing 80% of the total radioactivity with an R_F = 0.5 corresponding to that of N_3AMP. A minor component (20%) with an R_F = 0.38 was also present and probably represents a deactivation product of N_3AMP. The λ_{max} of the [^{14}C]N_3AMP sample was at 275 nm and, after photolysis, the spectrum resembled that of the photoproducts of N_3AMP. The specific radioactivity of the [^{14}C]N_3AMP used in the photoincorporation studies was expressed as dpm/nmol of photoactive component. The concentration of analogs was determined using millimolar extinction coefficients of 13.3 for N_3AMP [19] and 15.1 for BrAMP [20].

Assays in the presence of AMP analogs. When analogs of AMP were tested for their ability to activate phosphorylase, the assay (vide supra) was modified; bovine serum albumin which binds azido derivatives of nucleotides was not used to prevent denaturation and adsorption of phosphorylase onto glass surfaces during dilution to assay concentrations. The enzyme was first diluted to a concentration of approx. 1 mg/ml with a solution of 50 mM sodium glycerophosphate (pH 6.8) and 30 mM 2-mercaptoethanol. Further dilutions were made with the same buffer containing 0.8% glycogen. This procedure resulted in the retention of 75—80% of the original specific activity of the enzyme. Solutions of the analogs (50 μl) were added to aliquots of the enzyme (200 μl) from a Gilmont microsyringe; the enzymic reaction was initiated with 150 μl of the appropriate substrate mixture. The final concentration of the substrates in the assay was 0.9—1.0% glycogen and either 75 mM or 30 mM glucose 1-phosphate. 2—4 assays were carried out under a given condition together with two determinations of *V*. The activity of the enzyme in the pres-

ence of 75 mM glucose 1-phosphate, 1% glycogen, and 1 mM AMP (pH 6.8) was taken as the maximum velocity.

Dialysis equilibrium experiments. The non-covalent binding of [^{3}H]AMP to phosphorylase *a* was studied by equilibrium dialysis as previously described [21]. Each dialysis chamber contained two sectors which were loaded with 100 μl of protein or ligand solution. Experiments at a given concentration of [^{3}H]-AMP were run simultaneously in tripicate along with a control without enzyme. After 20—24 h at room temperature (23 ± 2°C), two aliquots (20 μl) were removed from each dialysis sector and delivered into glass counting vials. A volume of 0.2 ml of Protosol (New England Nuclear) was added followed by 10 ml of a toluene solution containing Omniflour (New England Nuclear). The vials were counted with a Beckman liquid scintillation system, Model LS-230.

Covalent incorporation of N_3AMP into phosphorylase. The irreversible binding of N_3AMP to phosphorylase *a* was determined by irradiating mixtures of the protein and N_3AMP at 23 ± 2°C with either a UVS-11 or a UVL-56 mineralight (Ultraviolet Products, Inc.) having a maximum emission at either 254 nm or 366 nm, respectively. An equilibration of 15 min in the dark preceded the irradiation. Solutions (1.5—3.0 ml) were photolyzed at a distance of 3—4 cm as a film of 1—2 mm in thickness for 15 min at 254 nm or in glass test tubes (1.3 × 10 cm) with gentle stirring for 45 min at 366 nm. Filtration through glass restricted the output of the 366 nm lamp to wavelengths above 300 nm. In mixtures containing 0.17 mM of both N_3AMP and adenosine, photoactivation of N_3AMP was 90—100% complete under the experimental conditions after 15 min at 254 nm or 30 min at 366 nm as judged by the change in absorption at 290 nm. Unirradiated samples remained in the dark during the period of photolysis. After irradiation the unbound photoproducts were separated from the protein by gel filtration on columns of Sephadex G-25 (1.5 × 28 cm). Fractions of 1.0—1.3 ml were collected. The absorbance of each fraction at 280 nm was determined and 50—100 μl were taken for determination of radioactivity by liquid scintillation counting (vide supra). A peak of radioactivity comigrated with the protein which was separated from the unbound radioactively labeled photoproducts. To insure that all of the radioactivity associated with the protein was covalently bound, aliquots of the protein (300—500 μl) were precipitated by the addition of 2 ml of an ice-cold solution of 5% trichloroacetate and 0.25% sodium tungstate [22] followed by another 2 ml of the trichloroacetate/tungstate solution containing 0.4 mg of bovine serum albumin. The precipitate was dissolved in 100 μl of 0.5 N NaOH and again precipitated. After two washes, the protein was dissolved in 1.0 ml of Protosol, 10 ml of an Omniflour/toluene mixture were added, and the radioactivity measured by liquid scintillation counting.

Results

Activation of phosphorylase b by N_3AMP

N_3AMP can substitute for AMP as an allosteric modifier of phosphorylase *b*. The pH vs. activity profile for the interaction of the enzyme with N_3AMP was determined between pH 6.5 and pH 7.5 and found to resemble that for AMP; maximum stimulation of glycogen synthesis occurs at pH 6.8. Enzymic assays

were carried out with 75 mM glucose 1-phosphate and 1% glycogen as substrates and varying concentrations of AMP, N_3AMP, or 8-bromoadenosine 5′-monophosphate (BrAMP). In Fig. 1, representative data are analyzed according to the method of Hill. At the highest concentration of N_3AMP which was tested (1.6 mM), phosphorylase *b* exhibited 68% of its activity with saturating levels of AMP. The apparent activation constant (K_a) for N_3AMP is approx. 33-fold greater than the K_a for AMP. BrAMP is much weaker than N_3AMP as an allosteric modifier of phosphorylase, at nucleotide concentrations of 1.6 mM, enzyme activity with N_3AMP is twice that observed with BrAMP. N_3AMP used in this experiment was prepared from the BrAMP sample and further purified by column chromatography on DEAE-cellulose. Since N_3AMP must contain less contaminating AMP than the starting compound, BrAMP, the increased level of activation by N_3AMP represents a specific interaction of the analog with the protein. By combining the data of Fig. 1 with another experiment over the same concentration range for both N_3AMP and AMP, maximal activation of phosphorylase *b* by N_3AMP estimated from a plot of reciprocal velocity versus reciprocal activator concentration is 87% of the value for AMP.

Heterotropic interactions are observed between the site for N_3AMP and other sites on the enzyme. The allosteric inhibitor, glucose 6-phosphate, increases the apparent K_a for both AMP and N_3AMP (Fig. 2). A comparison of Figs. 1 and 3 indicates that the activity of phosphorylase *b* with both AMP and N_3AMP is influenced by the substrate concentration. At each activator concentration, lowering the level of glucose-1-*P* from 75 mM to 30 mM decreases the observed activity; at 1.6 mM N_3AMP, the activity drops from 68% to 29% of *V*. The extrapolated value of K_a for N_3AMP at 30 mM glucose-1-*P* is 5 mM. The

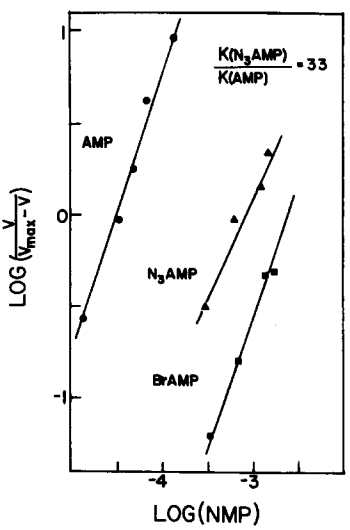
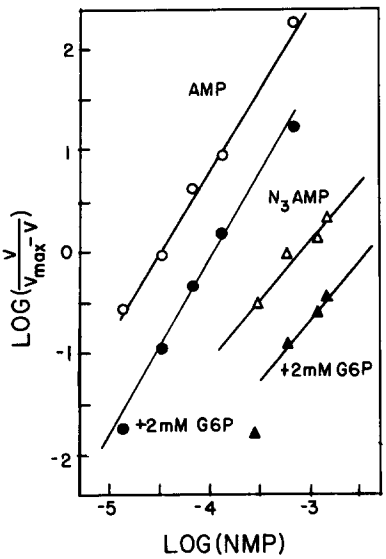


Fig. 1. Hill plot for the activation of phosphorylase *b* by nucleotide 5′-monophosphates with 75 mM glucose 1-phosphate and 1% glycogen as substrate. ●, AMP; ▲, N_3AMP; ■, BrAMP. Apparent K_a values are 30 μM for AMP, 1 mM for N_3AMP, and 2.6 mM for BrAMP. The S.D. of the data for N_3AMP and BrAMP are within the limits of the symbols used. Three of the points for AMP (●) with the following (*x*, *y*) coordinates have S.D. which exceed the limits of the symbols: (—3.85, 0.96 ± 0.03); (—4.15, 0.62 ± 0.07), and (—4.3, 0.25 ± 0.03).



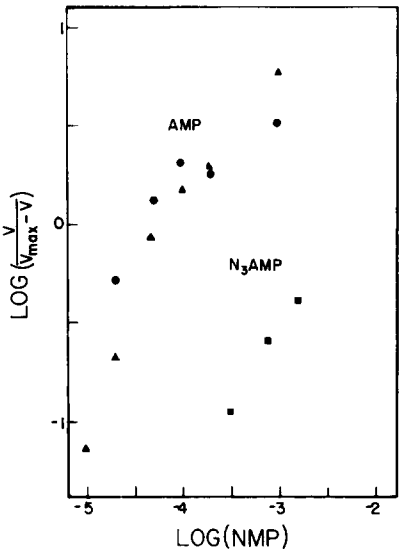


Fig. 2. Inhibition of phosphorylase *b* by glucose 6-phosphate with either AMP or N_3AMP as allosteric activator. ○, AMP; ●, AMP + 2.1 mM glucose 6-phosphate; △, N_3AMP; ▲, N_3AMP + 2.1 mM glucose 6-phosphate. The substrate mixture contained 75 mM glucose 1-phosphate and 0.9% glycogen. Apparent K_a values are 30 μM for AMP, 0.12 mM for AMP + 2.1 mM glucose 6-phosphate, 0.83 mM for N_3AMP, and 3.6 mM for N_3AMP + 2.1 mM glucose 6-phosphate. One point (○) for AMP with (x, y) coordinates of (—4.15; 0.62 ± 0.07) and one point (●) for AMP + 2.1 mM glucose 6-phosphate with (x, y) coordinates of (—3.15, 1.25 ± 0.11) have S.D. which exceed the limits of the symbols.

Fig. 3. A comparison of the ability of AMP (●, ▲) and N_3AMP (■) to activate phosphorylase *b* with 30 mM glucose 1-phosphate and 1% glycogen as substrates. The different symbols for AMP represent data from two separate experiments. The apparent K_a value for N_3AMP is 5 mM. Two points at an AMP concentration of 3 mM have S.D. which exceed the limits of the symbols, the (x, y) coordinates of the points with the S.D. of the measurements are (—3, 0.51 ± 0.15) and (—3, 0.78 ± 0.1). The S.D. of all other measurements are within the limits of the symbols.

behavior of the enzyme with AMP at 30 mM glucose-1-*P* is complex. The data (Fig. 3) are in good agreement with those of Madsen and Shechosky [23] for the activation of phosphorylase *b* by AMP at 24 mM glucose 1-phosphate. No attempt has been made to draw lines through experimental points of Fig. 3. The data are not extensive enough to delineate the shape of the Hill plot especially near the midpoint and to discriminate among the various models for allosteric control as outlined by Cornish-Bowen and Koshland [24].

The effects of N_3AMP and AMP on phosphorylase *b* are additive when each compound is present at a concentration below the apparent K_a (Table I). With 75 mM glucose 1-phosphate and concentrations of AMP up to 0.05 mM, 0.3 mM N_3AMP increases the activity of the enzyme: at saturating levels of AMP, N_3AMP has no effect.

Activation of phosphorylase a by N_3AMP

With either 30 mM or 75 mM glucose 1-phosphate as substrate, N_3AMP is capable of activating phosphorylase *a* to the same degree as saturating levels of AMP. Preparations of the enzyme used in this study exhibited 84% of their maximal activity in the absence of AMP. The results obtained with a glucose

TABLE I

EFFECT OF 0.3 mM N_3AMP ON THE AMP-INDUCED ACTIVATION OF PHOSPHORYLASE *b*

Specific activity is expressed as μmol of P_i/min per mg of protein. The substrates were 75 mM glucose 1-phosphate and 0.9% glycogen. The enzyme was diluted to assay concentrations without bovine serum albumin as described in Experimental procedure. This procedure resulted in an apparent loss of 20—25% of the original specific activity of the enzyme.

AMP (μM)	Specific activity	
	No additions	+0.3 mM N_3AMP
0	0	19.0
12.8	14.4	30.4
25.9	27.5	36.6
52.1	46.8	49.3
1000	60.5	60.3

1-phosphate concentration of 30 mM are presented in Table II. Because of the error in the assay (3—5%) and the relatively small extent of activation; K_a cannot be accurately determined from these data. K_a is of the order 30—100 μM, and much lower than the K_a for the interaction of N_3AMP with phosphorylase *b* (5 mM). Thus, N_3AMP resembles the natural activator in having a higher affinity for the *a* form of the enzyme.

Photoincorporation of N_3AMP into phosphorylase

Photoinduced irreversible binding of the analog was investigated with [^{14}C]-N_3AMP. In order to establish optimum conditions for the covalent incorporation, many variables were tested including protein concentration, N_3AMP concentration, time, temperature, and distance from a light source with maximum intensity either at 254 nm or 366 nm. The binding of [^{3}H]AMP to irradiated phosphorylase *a* was also measured. The highlights of these studies may be summarized in the following statements: (a) Short periods of exposure (2—15 min) at 254 nm led to incorporation of N_3AMP. Although there was no change in the affinity of the photolyzed enzyme for AMP, a progressive decrease in

TABLE II

N_3AMP ACTIVATION OF PHOSPHORYLASE *a*

Assays were carried out with 30 mM glucose 1-phosphate and 1% glycogen, pH 6.8. A concentration of 1 mM AMP is at least 1000-fold greater than that required for full activation of phosphorylase *a*. Specific activity is expressed as μmol of phosphate/min per mg of protein. Numbers in parenthesis are S.D. of the measurements.

AMP concn. (mM)	N_3AMP concn. (mM)	Trials	Specific activity	Relative activity
1	0	6	80 (2)	100
0	0.4	2	80.9 (0.6)	100
0	0.115	2	75.6 (0)	95
0	0.029	4	72.8 (0.7)	91
0	0.012	4	72.6 (0.8)	91
0	0.001	4	67.5 (0.4)	84
0	0	4	67 (1)	84

enzymic activity was observed; 50% of the original activity remained after 10 min. (b) N_3AMP was photoactivated by light at 366 nm under conditions where the enzyme retained a strong affinity for AMP and 90—100 of its maximum activity. Long periods of exposure (30—45 min) were required and the incorporation of N_3AMP was comparable to or less than that achieved by light at 254 nm.

In order to establish that incorporation of [^{14}C]N_3AMP into phosphorylase *a* represents true photoaffinity labeling as defined by Ruoho et al. [25], experiments were carried out with and without the addition of 2-mercaptoethanol as a scavenger (Table II). N_3AMP reacts very slowly with 2-mercaptoethanol at neutral pH in the dark but, upon irradiation, 8-aminoadenosine 5′-monophosphate is rapidly formed [26]. The function of the scavenger is to destroy activated molecules of N_3AMP which exist in solution and, thereby, prevent nonspecific reactions with the protein [25]. Mixtures of 31 μM protein, 177 μM [^{14}C]N_3AMP and a 100-fold excess of 2-mercaptoethanol over protein were irradiated as a thin film (1—2 mm) for 15 min at 23 ± 2°C with a light source having a maximum intensity at 254 nm. Phosphorylase was labeled with 0.119 ± 0.006 mol N_3AMP/mol monomer in the presence of 13 mM 2-mercaptoethanol; in the absence of scavenger, the average value of $\bar{n}$ was 0.252 ± 0.004. These values were measured by analysing four samples in each experiment. The data are consistent with a maximum of 10% saturation of a single unique binding site for N_3AMP or a lower degree of saturation for multiple binding sites.

Specificity of the labeling was further investigated by testing the ability of the natural ligand, AMP, to compete with N_3AMP for the nucleotide binding site (Table III). Since AMP absorbs ultraviolet light, the irradiated mixtures of protein and [^{14}C]N_3AMP to be compared contained either AMP or adenosine, a compound which does not bind to the site for AMP on phosphorylase [2]. Without irradiation, very little N_3AMP is bound to the protein (Expt. 1a). Incorporation of 0.08 mol of N_3AMP/mol of monomer is induced by illumination with short-wavelength ultraviolet light (Expt. 1b); addition of a 1.2-fold excess of AMP over N_3AMP reduces the apparent covalent binding of the

TABLE III

COVALENT INCORPORATION OF [^{14}C]N_3AMP INTO PHOSPHORYLASE *a*. EFFECT OF AMP UNDER TWO DIFFERENT CONDITIONS OF IRRADIATION

The solvent contained 20 mM sodium phosphate, 0.4 M KCl, and 13 mM 2-mercaptoethanol, pH 7.1. Other experimental details are given in Experimental procedure. The average value of $\bar{n}$ is expressed together with the S.D. of the measurements. The total number of samples analyzed is indicated in parentheses.

Expt.	Protein (μM)	Light source (nm)	Exposure time (min)	N_3AMP (μM)	Adenosine (μM)	AMP (μM)	$\bar{n}$
1a	34	—	—	131	154	—	0.005 ± 0.001 (4)
1b	34	254	15	131	154	—	0.085 ± 0.004 (4)
1c	34	254	15	131	—	153	0.054 ± 0.003 (3)
2	29	366	45	117	152	—	0.07 ± 0.01 (6)
3	30	366	45	122	—	151	0.023 ± 0.003 (3)
4	37	366	45	218	272	—	0.11 ± 0.01 (2)

analog by about 40% (Expt. 1c). Complete reversal of the incorporation of the N_3AMP is not expected since the activator site is not saturated at 154 μM AMP. The results of equilibrium dialysis of phosphorylase *a* (34 μM) vs. [^{3}H]AMP under conditions of Expt. 1 of Table III (data not shown) indicate that approximately 65% of the binding sites for AMP are occupied by the effector in the absence of N_3AMP. The long-wavelength ultraviolet light source was used for the next series of experiments. Under comparable conditions, the magnitude of the incorporation at 366 nm (Expt. 2) is only slightly lower than that observed at 254 nm (Expt. 1b). When AMP is present during irradiation (Expt. 3), incorporation of 0.05 mol of N_3AMP/mol of monomer which accounts for approx. 70% of the light-dependent incorporation observed in Expt. 2 is prevented. Increasing the protein and N_3AMP concentration results in an increased level of incorporation of N_3AMP as illustrated by Expt. 4.

Discussion

N_3AMP is a suitable photoaffinity probe for the allosteric site on phosphorylase. N_3AMP mimics AMP as a modifier of phosphorylase activity. The analog activates both forms of the enzyme in the absence of AMP and displays heterotropic interactions with the substrate, glucose 1-phosphate, and the allosteric inhibitor, glucose 6-phosphate. The additivity of the effects of low concentrations (less than the K_a) of N_3AMP and AMP with 75 mM glucose 1-phosphate as substrate reinforces the view that both nucleotides promote the active conformation of the enzyme. The activitation data for both phosphorylases *a* and *b* indicate that N_3AMP has a lower affinity than AMP for the nucleotide binding site. N_3AMP can be covalently incorporated into the protein by long-wavelength ultraviolet light under conditsons which do not significantly alter either the enzymic activity or the binding of AMP. Irradiation with short-wavelength ultraviolet light leads to higher levels of covalent attachment and, although the enzymic activity is decreased, the affinity of phosphorylase for AMP is unchanged.

The incorporation of N_3AMP into phosphorylase conforms to the following criteria outlined by Ruoho et al. [25] for true photoaffinity labeling: (a) The protein is labeled in the presence of high concentrations of a scavenger capable of reacting with unbound photogenerated intermediates of N_3AMP. (b) Covalent attachment of N_3AMP is negligible without irradiation, and (c) light-dependent incorporation of the analog is reduced in the presence of the natural ligand, AMP. Studies of the crystallographic structure of phosphorylase *a* reveal only one nucleotide binding site per monomer at AMP concentrations up to 0.5 mM [2]. Reversal of N_3AMP binding by AMP at 0.15 mM supports the view that both ligands can occupy the nucleotide-specific site. When the total light-induced incorporation of N_3AMP is in the range of $\overline{n} = 0.065$—0.08, N_3AMP labels at least 3—5% ($\overline{n} = 0.03$—0.05) of the AMP binding sites. Some or all of the N_3AMP incorporation observed with AMP present may also occur at the activator site since, at 0.15 mM AMP, about 35% of these sites remain free. The explanation for the higher degree of AMP-reversible incorporation observed at 366 nm as compared to that at 254 nm is not known. Perhaps, the loss of enzymic activity which occurs during illumination at 254 nm is accompanied

by the unmasking of hydrophobic sites on the surface of the protein which bind AMP and N_3AMP with equal avidity.

In future experiments, uncertainties regarding the physical structure of the enzyme will be minimized by illuminating the protein/N_3AMP mixtures with long-wavelengh ultraviolet light (greater min than or equal to 300 nm). Higher concentrations of AMP can be used to test reversal of N_3AMP incorporation since AMP has negligible absorption above 300 nm. In order to increase the total incorporation of N_3AMP, the protein will be irradiated in several cycles with separation of unbound photoproducts between cycles. This approach as well as separation of labeled and unlabeled enzyme by affinity chromatography on columns of immobilized AMP should provide quantities of modified protein suitable for physical and chemical studies.

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