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Adenosine 5'-(2-Bromoethyl)-phosphate

A NEW AFFINITY LABEL FOR ADENINE NUCLEOTIDE SITES IN PROTEINS*

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A new adenosine analogue adenosine 5'-(2-bromoethyl)-phosphate has been synthesized. The reactive moiety, a bromoalkyl group, has the ability to react with the nucleophilic side chains of several amino acids. This compound reacts with NAD-dependent isocitrate dehydrogenase from pig heart, causing inactivation. Addition of the allosteric regulator ADP to the reaction mixture protects the enzyme from loss of activity. A second adenosine analogue has been synthesized, adenosine 5'-(*n*-propyl)-phosphate, which is used to assess any effects that might arise from the noncovalent interaction of adenosine 5'-(2-bromoethyl)-phosphate with the enzyme. It is proposed that adenosine 5'-(2-bromoethyl)-phosphate reacts with an adenine nucleotide site on isocitrate dehydrogenase and that this compound may have general applicability as an affinity label of catalytic and regulatory adenine nucleotide sites in proteins.

Purine nucleotides, particularly adenine nucleotides, act as coenzymes and regulators in a wide variety of biochemical reactions. In fact, regulation by adenine nucleotides is so prevalent that Atkinson and Walton proposed the adenylate control hypothesis, stating that energy metabolism is sensitively controlled by the varying distribution among AMP, ADP, and ATP of the total adenylate pool (1). One approach to probing the structure of the purine nucleotide sites of proteins is to use nucleotide analogues which simulate the natural coenzyme or effector, but which have reactive functional groups at particular positions of the purine or ribose ring. Several such purine nucleotide analogues have been synthesized. Colman *et al.* (2) and Pal *et al.* (3) have described the synthesis and applications of a class of purine nucleotide analogues carrying *p*-fluorosulfonylbenzoyl groups. One of the most useful of these derivatives has been 5'-*p*-fluorosulfonylbenzoyl adenosine, which has been found to chemically modify a variety of proteins including glutamate dehydrogenase (4), malate dehydrogenase (5), RNA polymerase (6), pyruvate kinase (7), phosphofructokinase (8, 9), cyclic AMP-dependent protein kinase (10, 11), mitochondrial F_1 ATPase (12), and an ADP binding protein of human platelet membranes (13). Hampton (14) has described a number of purine nucleotide analogues such as N^6-*p*-fluorobenzoyl adenosine phosphates, 6-chloropurine ribose phosphates, and carboxylic-phosphoric anhydrides of adenine nucleotides. Experience with these and other analogues has been reviewed (15). Despite the effectiveness of some of these analogues, their use entails certain disadvantages. The compounds may be relatively water insoluble like the *p*-fluorosulfonylbenzoyl adenosines; they may be very unstable like carboxylic-phosphoric anhydrides of nucleosides; they may carry bulky groups which may weaken the prior binding of the reagent; or the reactive group may be at a region where major classes of enzymes using adenine nucleotides are not expected to have catalytically significant functional groups. We have synthesized an adenosine analogue, adenosine 5'-(2-bromoethyl)-phosphate, which is water-soluble, is sterically very similar to adenosine diphosphate, has a negative charge at neutral pH, and is stable over a wide range of pH and buffer conditions. In addition, the bromoalkyl group is capable of reaction with most nucleophilic groups in proteins and is located at a position where phosphotransferase, pyrophosphotransferase, and nucleotidyltransferase enzymes may be expected to have catalytically important amino acid residues. In this paper, we present evidence suggesting that adenosine 5'-(2-bromoethyl)-phosphate reacts covalently at an adenine nucleotide site of the NAD-dependent isocitrate dehydrogenase from pig heart.

EXPERIMENTAL PROCEDURES

Materials—Bromoethanol was purchased from Aldrich Chemical Co., but was purified prior to use in the synthesis of 5'-BrEtAMP.[1] To 100 g of bromoethanol, anhydrous sodium carbonate was added until the suspension was neutral to pH paper. Anhydrous sodium sulfate (14 g) was then added and the whole suspension was allowed to stand overnight before being filtered. Several grams of Drierite (W. A. Hammond Drierite Co.) were added to the filtrate and the suspension was allowed to stand for several hours in a dry place. The mixture was filtered again and the filtrate was distilled under reduced pressure. Only those fractions distilling at 59–60°C were collected and stored over dry nitrogen. Pyridine was distilled over NaOH and stored over 4-Å molecular sieves. NAD-dependent isocitrate dehydrogenase was prepared according to Ramachandran and Colman (16). Enzyme was dialyzed against 0.05 M 1,4-piperazinediethanesulfonic acid buffer, pH 7.0, containing 2 mM $MnSO_4$ and 20% glycerol, before use. For native enzyme, the protein concentration was determined by multiplying the absorbance at 280 nm by 1.55 (17).

Isocitrate, ADP, NAD, 5'-adenosine phosphomorpholidate, and snake venom phosphodiesterase were all obtained from Sigma Chemical Co. Dioxane/HCl was purchased from Pierce Chemical Co.

Preparation of Adenosine 5'-(2-Bromoethyl)-phosphate—One millimole of 5'-adenosine phosphomorpholidate was dissolved in 25 ml of dry pyridine and was evaporated to dryness. This procedure was repeated three times, after which 25 ml of dry pyridine were added, followed by 3.5 ml (50 mmol) of 2-bromoethanol and 0.25 ml of 4 N dioxane/HCl. The mixture was allowed to stand overnight and 25 ml of water were added to the reaction mixture. The solvent was evaporated *in vacuo* to a glassy residue. To precipitate the solid, 100 ml of ethanol:acetone (1:1) were added. The precipitate containing crude 5'-BrEtAMP was collected, redissolved in water, and concentrated.

To purify the 5'-BrEtAMP, the concentrate was applied to a column (1.5 × 24 cm) of the hydrogen form of AG50-X8 (Bio-Rad Laboratories) and was eluted with water. The fractions were examined

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[1] The abbreviations used are: 5'-BrEtAMP, adenosine 5'-(2-bromoethyl)-phosphate; and 5'-*n*-PrAMP, adenosine 5'-(*n*-propyl)-phosphate.

by thin layer chromatography, as described under "Results." The major ultraviolet-absorbing peak occurred at 225 ml; and fractions eluting between 100 and 325 ml were found to contain chromatographically homogeneous 5'-BrEtAMP. These fractions were pooled, titrated to neutrality with NaOH, and evaporated to dryness. (Samples used for elemental analysis were not neutralized prior to evaporation.) Approximately 0.21 mmol of 5'-BrEtAMP were recovered in this peak. Fractions eluting after 400 ml contained 5'-AMP as the major product.

Determination of Bromide Content of 5'-BrEtAMP—BrEtAMP was hydrolyzed by 0.6 M NaOH in a boiling water bath for 2 h, after which the solution was neutralized by the addition of glacial acetic acid. The bromide which was released was measured using a Beckman pH meter equipped with a bromide electrode and standard bromide solutions containing the same salt concentration as the sample.

Preparation of Adenosine 5'-(n-Propyl)-phosphate—One millimole of 5'-AMP, $2H_2O(2H^+)$ was dissolved in 250 ml of *n*-propyl alcohol and 2 mmol of triethylamine were added. Dicyclohexylcarbodiimide (5 mmol) was added and the mixture was stirred for 72 h, a time period sufficient to allow almost complete conversion of the 5'-AMP to product, as judged by thin layer chromatography. The solvent was evaporated *in vacuo* and the residue was dissolved in 25 ml of water. The insoluble dicyclohexylurea was removed by filtration and the filtrate was extracted twice with 15 ml of ether. The aqueous solution was adjusted to neutrality with NaOH and was evaporated to dryness.

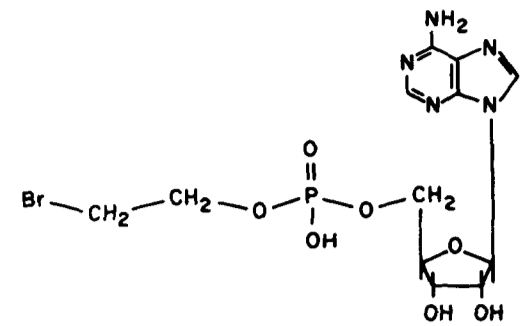
Enzyme Assay—Isocitrate dehydrogenase activity was determined at 25°C in Tris, 0.033 M acetate buffer, pH 7.2, by measuring the increasing absorbance of NADH at 340 nm using a Gilford model 240 spectrophotometer equipped with an expanded scale recorder (0 to 0.1 absorbance full scale). The substrate concentrations used were 20 mM isocitrate, 1 mM NAD^+, and 1.33 mM $MnSO_4$ in a total volume of 1.0 ml.

Kinetics of Reaction of 5'-BrEtAMP with NAD-dependent Isocitrate Dehydrogenase—Isocitrate dehydrogenase (0.217 mg/ml) was incubated at 25°C in 0.1 M triethanolamine acetate, pH 8.0, containing 20% glycerol, 5 mM magnesium sulfate, and 125 μM EDTA for 90 min, after which the nucleotide analogues were added to initiate the reaction. When ADP was present, it was added after the preincubation period. During the course of reaction of isocitrate dehydrogenase with 5'-BrEtAMP, aliquots were withdrawn at given time intervals and assayed by the procedure described above. Since the reagent was diluted approximately 200-fold in the assay solution, it was considered that essentially no reaction between 5'-BrEtAMP and isocitrate dehydrogenase took place during the 1- to 2-min period required to conduct the assay. Indeed, the individual assays were linear and the measured rates were assumed to reflect the residual enzymatic activity at the time of withdrawal of aliquots from the reaction mixture.

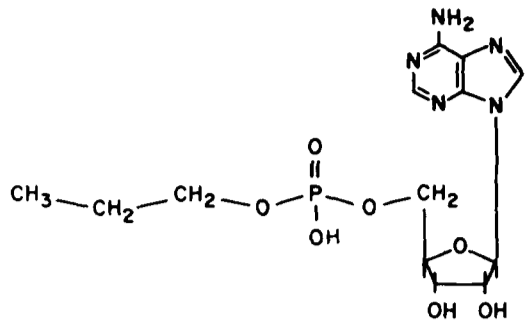
RESULTS AND DISCUSSION

Characterization of Adenosine 5'-(2-Bromoethyl)-phosphate—The purity of the compound synthesized was assessed by high pressure liquid chromatography and thin layer chromatography with several solvent systems. Using a Varian model 5000 high pressure liquid chromatograph, the sample was chromatographed on a reverse phase column (MCH-10, 300 × 4 mm) using 0.1 M triethylammonium acetate, pH 6.9, 20% methanol (flow rate 2.0 ml/min) as mobile phase. A single peak was observed with a retention time of 9.9 min. Thin layer chromatography was performed on cellulose-coated aluminum plates (Brinkmann, 0.1-mm thickness) using the following solvent systems: 1) ethanol, 1 M ammonium acetate, 0.5 M trichloroacetic acid (60:30:10); 2) acetonitrile, 2 M lithium chloride, water (70:15:15); 3) methyl ethyl ketone, acetone, water (65:20:15); and 4) *t*-amyl alcohol, 88% formic acid, water (40:40:20). In all four cases, a single ultraviolet absorbing spot was observed, exhibiting R_F values of 0.66, 0.66, 0.34, and 0.88 in solvents 1, 2, 3, and 4, respectively. For comparison, 5'-AMP exhibits R_F values of 0.40, 0.15, 0.07, and 0.58 in solvents 1, 2, 3, and 4, respectively.

The 5'-BrEtAMP has been demonstrated to have the structure shown in Fig. 1 on the basis of the ultraviolet absorption spectrum, elemental analysis, digestion with phosphodiesterase, and ^{13}C and ^1H NMR spectra. The ultraviolet absorption spectrum exhibits a maximum at 259 nm and minimum at 227 nm when measured in water at pH 7. The 250:260 and 280:260 ratios along with maxima and minima at different values of pH are almost identical with those of 5'-AMP.



Adenosine 5'-(2-Bromoethyl)-Phosphate



Adenosine 5'-(n-Propyl)-Phosphate

FIG. 1. **Structures of adenine nucleotide analogues.**

The elemental analysis is in agreement with the structure given in Fig. 1, assuming that 1 water molecule is associated with the compound.

$C_{12}N_5O_8H_{19}P_1Br_1$

Calculated:	*30.53C 14.83N 4.03H*
Found:	30.98C 13.82N 4.01H

Phosphate was determined by the method of Fiske and Subbarow (described in Ref. 18). The ratio of phosphate to spectrophotometrically determined adenosine was 1.01:1.00. The bromide content was measured after hydrolysis in 0.6 N NaOH, as described under "Experimental Procedures." The ratio of Br^- to spectrophotometrically determined adenosine was 1.06:1.00. Upon digestion with snake venom phosphodiesterase, 5'-BrEtAMP yielded a single ultraviolet absorbing material having R_F values identical with those of 5'-AMP in the four solvent systems described above.

All ^{13}C NMR spectra were obtained using a Bruker HFX-90. Proton-decoupled ^{13}C NMR spectra were measured in 50% deuterium oxide and the resonance peaks were assigned by comparison with those of standard adenosine phosphates (19) and 2-bromoethanol. The resonance peaks were assigned as follows: C_6 at 155.6 ppm, C_2 at 152.9 ppm, C_4 at 150.2 ppm, C_8 at 139.9 ppm, C_5 at 118.8 ppm, $C_{1'}$ at 87.4 ppm, $C_{4'}$ at 84.1 ppm (doublet), $C_{2'}$ at 74.4 ppm, $C_{3'}$ at 70.9 ppm, $C_{5'}$ at 65.9 ppm, C_1 of the bromoethanol moiety at 65.9 ppm, C_2 of the bromoethanol moiety at 31.6 ppm (doublet). The phosphodiester nature and the proposed structure was also evident from coupling constants of phosphorus and carbon observed in the spectrum. The fine structures of P-O-C peaks were not ob-

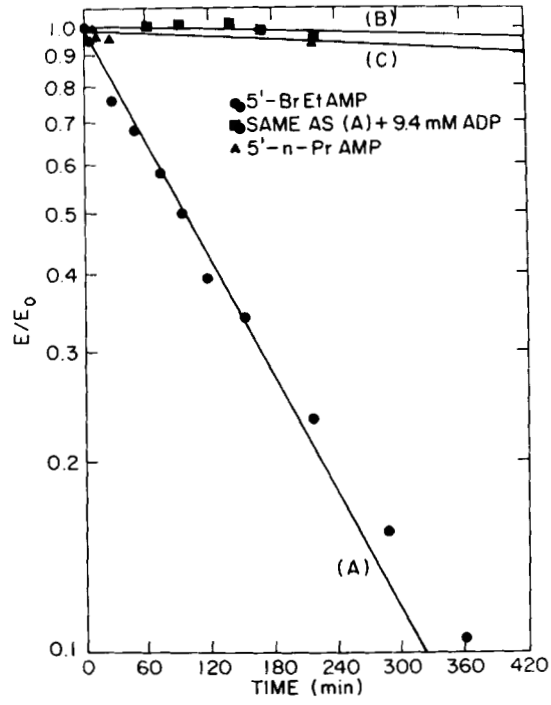


FIG. 2. **Reaction of adenosine nucleotide analogues with NAD-dependent isocitrate dehydrogenase from pig heart.** Isocitrate dehydrogenase (0.217 mg/ml) was incubated with the nucleotide analogues at 25°C in 0.1 M triethanolamine acetate buffer, pH 8.0, containing 20% glycerol, 5 mM magnesium sulfate, and 125 μM EDTA. At each indicated time, an aliquot was withdrawn and assayed as described under "Experimental Procedures." *A*, 12 mM 5'-BrEtAMP added; *B*, same as (*A*) + 9.4 mM ADP added to the reaction mixture; *C*, 16.0 mM 5'-*n*-PrAMP added.

served, which is consistent with the general experience that P-O-C coupling constants are generally smaller than P-O-C-C coupling constants in cases of 5'-nucleotides (19).

All proton NMR spectra were measured in a Perkin-Elmer R-12 (60 MHz). The spectrum contains two clearly resolvable peaks at δ8.30 and δ8.05, which are assigned to H_2 and H_8 protons, and another clearly resolved doublet at δ6.0, which is assigned to $H_{1'}$. The rest of the spectrum is consistent with the structure shown in Fig. 1.

Characterization of 5'-(n-Propyl) Adenosine Monophosphate—The purity of the compound was assessed by thin layer chromatography on cellulose using the four solvent systems described above. The ultraviolet absorption spectra at different pH values are remarkably similar to those of 5'-BrEtAMP and 5'-AMP spectra, as expected.

The proton NMR spectrum, measured in deuterium oxide, gave peaks which are assigned as follows: H_2 or H_8, δ8.30; H_2 or H_8, δ8.05; $H_{1'}$, δ6.00 (doublet); $H_{2'}$, $H_{3'}$, $H_{4'}$, and $H_{5'}$, combined multiplets, and HDO, δ4.00 to 4.50; H_1 of propanol, multiplet centered at δ3.6; H_2 of propanol, multiplet centered at δ1.45; H_3 of propanol, triplet centered at δ0.65.

Reaction of 5'-BrEtAMP with NAD-dependent Isocitrate Dehydrogenase from Pig Heart—The pig heart NAD-dependent isocitrate dehydrogenase is an allosteric enzyme which is activated by ADP (20). The enzyme has been shown to bind both NAD and ADP (21) and thus was expected to be a good candidate for affinity labeling by the adenine nucleotide analogue, 5'-(2-bromoethyl) adenosine monophosphate. Incubation of the NAD-dependent isocitrate dehydrogenase with 5'-BrEtAMP in 0.1 M triethanolamine acetate, pH 8.0, containing 5 mM magnesium sulfate, 20% glycerol, and 125 μM EDTA, results in a progressive loss of isocitrate dehydrogenase activity, as shown in Fig. 2, *line A*. The time-dependent inactivation follows pseudo-first order kinetics as far as about 15% of initial activity, yielding a rate constant of 0.0092 min^{-1} (an average of several determinations) at a 5'-BrEtAMP concentration of 12 mM.

The rate constant for inactivation of isocitrate dehydrogenase was measured over the concentration range of 4 to 60 mM 5'-BrEtAMP, revealing a nonlinear dependence of the rate constant on the reagent concentration. These data are consistent with a mechanism in which 5'-BrEtAMP binds reversibly to the enzyme (K_d = 50 mM) prior to the irreversible inactivation. Such kinetic behavior is normally expected for an affinity label.

In order to evaluate the contribution of the AMP moiety to the effectiveness of 5'-BrEtAMP in inactivating isocitrate dehydrogenase, the effect of 2-bromoethanol on the enzyme was tested. Isocitrate dehydrogenase, upon incubation with 36 mM 2-bromoethanol under the same conditions as those of Fig. 2, was very slowly inactivated. The reaction obeyed pseudo-first order kinetics with an observed rate constant of 0.00064 min^{-1}, yielding a calculated second order rate constant for the 2-bromoethanol reaction of 0.018 min^{-1} M^{-1}. Thus, at 12 mM 2-bromoethanol, the rate for inactivation would be 1/43 that observed for 5'-BrEtAMP at the same concentration.

To assess the possibility that inactivation by 5'-BrEtAMP resulted from a destabilization of the enzyme by noncovalent binding of this compound, the effect on the enzyme of incubation with the nonreactive 5'-(*n*-propyl) AMP was tested. Fig. 2, *line C* shows that 5'-*n*-PrAMP does not inactivate the enzyme, thus implying it is the bromoalkyl group that is responsible for the inactivation caused by that reagent.

Specificity of a protein chemical modification reaction can be indicated by the ability of a particular ligand to markedly reduce the reaction rate of the enzyme with the reagent. Fig. 2, *line B* demonstrates that the inclusion of 9.4 mM ADP in the reaction mixture completely abolishes the inactivation, suggesting that the ADP site may be the site of attack by 5'-BrEtAMP.

The importance of the adenosine moiety in the reaction, the specific protection offered by the allosteric effector ADP, and the inability of adenosine 5'-(*n*-propyl)-phosphate to inactivate the enzyme all indicate that 5'-BrEtAMP may be acting as an affinity label of an adenine nucleotide site in the NAD-specific isocitrate dehydrogenase. It is proposed that adenosine 5'-(2-bromoethyl)-phosphate may have general applicability as an affinity label for proteins which have either catalytic or regulatory adenine nucleotide binding sites.

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