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Synthesis and Preliminary Characterization of a New Photoaffinity Labeled Adenosine Nucleotide

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SUMMARY

4-Azido-2-nitrophenyl adenosyl pyrophosphate (ANAP), a new photoaffinity labeled adenosine nucleotide, was synthesized by coupling 4-azido-2-nitrophenyl phosphate and adenosine 5'-monophosphomorpholidate. Radioactive analogues were prepared from either [^{14}C]-AMP or iodination of 4-azido-2-nitrophenol. ANAP was shown to be a competitive inhibitor of glucose-6-phosphate dehydrogenase against NADP with an inhibition constant of 9.5 μM. Iodination of ANAP did not affect its inhibition. Thus ANAP is a good photoaffinity analogue of NADP. It probably will also serve as an analogue of ATP or ADP.

Key Words: Photoaffinity Labeling, NAD/NADP analogue, ATP/ADP analogue, adenosine nucleotide label

INTRODUCTION

Photoaffinity labels have been used extensively for the study of the location, structure and function of ligand binding sites of diverse proteins [1,2]. Many different photoactivatable compounds have been synthesized for various purposes. Of the adenosine nucleotide analogues, almost all of the photoactivatable groups are located at the adenosine portion of the molecule, for example, the 8-azido analogues of AMP, cAMP, ADP and ATP [3]. Such modifications may in some cases affect the binding of the label to proteins since the adenosine moiety usually serves as an

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anchor of the molecule [4]. In order to preserve the binding domain and for the purpose of probing parts of a protein other than the nucleotide binding site, we have designed and synthesized a new adenosine nucleotide analogue that can be used for the study of NAD, NADP, ADP and ATP binding proteins.

MATERIALS AND METHODS

Materials. All the materials used for the synthesis were of reagent grade. Aldrich Chemical Company supplied morpholine, pyridine, sodium nitrite, 4-amino-2-nitrophenol, phosphorous oxychloride, dicyclohexyl carbodiimide and thin layer silica gel plates. Sigma Chemical Company supplied adenosine 5'-monophosphomorpholidate, AMP and DEAE-Sephadex A-25-120. Radioactive [U-^{14}C]-AMP was obtained from Dupont, NEN Products.

Synthesis. ANAP was prepared as shown in Figure 1 by coupling 4-azido-2-nitrophenyl phosphate with adenosine 5'-monophosphomorpholidate. 4-Azido-2-nitrophenyl phosphate was synthesized by diazotization and azide replacement of the amino group of 4-amino-2-nitrophenol [5] followed by phosphorylation with $POCl_3$ [6] as described below.

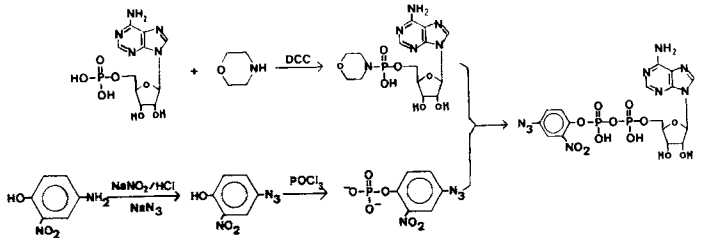

Figure 1. Synthetic scheme for 4-azido-2-nitrophenyl adenosyl pyrophosphate (ANAP).

To 12.5 ml of 0.8 molar solution of 4-amino-2-nitrophenol in 3.5 N HCl at -10°C (Methanol/ice) was added dropwise with stirring 2.5 ml of 4.4 M aqueous sodium nitrite solution. After the addition was complete, the solution was allowed to warm up to 0°C

in the dark and 10 ml of ethanol was added followed by dropwise addition of 2.5 ml of 3.7 M sodium azide. The solution was further stirred for 30 minutes in the dark and then evaporated under vacuum to a residue which was extracted three times with 10 ml portions of diethyl ether. The ether extract was back washed with 10 ml of water and dried over anhydrous sodium sulfate. The final solution was evaporated to dryness under vacuum dissolved in minimum volume of heptane and allowed to stand at -20°C to crystallize. The product showed an IR (Nujol) spectrum characterized by the azido band at 2150 cm^{-1}, and UV absorption maxima at 247 and 386 nm in methanol. Proton-NMR spectrum gave all the characteristics of the compound as indicated by Lauquin et al. [5].

Phosphorylation of 4-azido-2-nitrophenol according to the procedures of Symons [7], and Maassen and Möller [8] gave very poor yields. The synthesis was carried out with $POCl_3$ in pyridine [6]. To the crystallized 4-azido-2-nitrophenol (0.18 g) dissolved in one ml anhydrous dimethoxyethane was added one mmol of freshly distilled phosphorous oxychloride. The solution was refluxed as one mmol of dry pyridine was added dropwise and further refluxed for another hour. After evaporation to dryness under vacuum, the residue was added 20 ml pyridine/water (1:10) and stirred for 2 hours. Unreacted 4-azido-2-nitrophenol which precipitated was removed by filtration. The filtrate was condensed under vacuum and dissolved in water. The product, 4-azido-2-nitrophenylphosphate, was purified on a DEAE-Sephadex A-25 column (1.5 x 28 cm) with a linear gradient of 0.01 M/0.4 M triethylammonium bicarbonate, pH 7.4. The compound eluted at 0.25 M gave an absorption spectrum identical to that reported by Lauquin et al. [5], and had an IR band at 2150 cm^{-1} which diminished on irradiation. It was converted to its pyridinium salt by repeated evaporation in vacuum several times after addition of dry pyridine. To this was added 0.3 mmol of adenosine 5'-monophosphomorpholidate. The

mixture was freed of water moisture by repeated evaporation after addition of anhydrous pyridine and finally suspended in 2 ml anhydrous pyridine. The solution was left standing in a desiccator in the dark at room temperature. After several days, the reaction was terminated by addition of water and evaporation to a syrup. The desired product was purified on a DEAE-Sephadex column (eluted at 0.3 M) with a linear gradient of 0.01 M/0.4M triethylammonium bicarbonate, pH 7.4. The product, ANAP migrated as a single spot on a silica gel TLC plate with an Rf value of 0.79 (ammonium acetate:ethanol=3:7) whereas the Rf value for 4-azido-2-nitrophenylphosphate was 0.66. The overall yield was 14%. ^{31}P-NMR (D_2O) showed two pairs of doublets at -10.55 and -10.76 ppm, and -16.43 and -16.64 ppm for the P_α and P_β, respectively. Proton NMR showed all the characteristics for the phenolic as well as the adenosyl moiety of the compound. IR (Nujol) spectrum revealed a strong azido band at 2150 cm^{-1}, which diminished on ultraviolet irradiation. The molar absorptivity was determined to be 39.7 x 10^3 M^{-1} cm^{-1} at 260 nm in 50 mM potassium phosphate buffer, pH 7.0.

Radioactive [U-^{14}C]ANAP was synthesized from [U-^{14}C]AMP after converting it to [U-^{14}C]adenosine 5'-monophosphomorpholidate (Figure 1) [9]. To a 0.2 ml solution of ten microcuries of [U-^{14}C]AMP (Specific activity 507 mCi/mmol) and 10 µmol of AMP triethylammonium salt in 50% tertiary butyl alcohol was added 20 µmol of freshly distilled morpholine. The solution was refluxed while 40 µmol of dicyclohexylcarbodiimide in tertiary butyl alcohol was added dropwise over a period of 4 hours. After addition, the solution was further refluxed for another half an hour. The dicyclohexyl urea precipitate was removed on cooling and the solution condensed under vacuum. The residue was dissolved in 2 ml water and extracted three times with 2 ml ether. The aqueous solvent was then replaced with pyridine by repeated

evaporation after addition of 2 ml anhydrous pyridine. The final solution of [U-^{14}C]adenosine 5'-monophosphomorpholidate was used to react with 4-azido-2-nitrophenylphosphate as mentioned above. The specific radioactivity of the final product was 1 mCi/mmol.

For the preparation of iodinated ANAP, 4-azido-2-nitrophenol was iodinated by the iodine monochloride method [10] in 0.5 M Na_2HPO_4, pH 10.8. Based on the iodination studies of phenolic compounds such as tyrosine [11], the site of iodine introduction is presumably position 6 of the benzene ring, ortho to the free hydroxyl group. The iodinated compound was then further phosphorylated and condensed with adenosine 5'-monophosphomorpholidate as described above to form the desired iodinated ANAP.

Assays. Glucose-6-phosphate dehydrogenase was assayed spectrophotometrically at 30°C according to Haghighi and Levy [12]. The assay mixture contained 6.7 mM $MgCl_2$, 1.2 mM glucose-6-phosphate, 0.1 mM NADP and appropriate enzyme concentration in 83 mM triethanolamine HCl, pH 7.6. For inhibition studies, the assay mixture also contained ANAP as indicated in the figure legend.

RESULTS AND DISCUSSION

Synthesis. The synthesis of ANAP is straightforward. To obtain high yields of the product, the reaction medium must be free of water moisture. ANAP is fairly stable and can be purified by liquid chromatography as shown in Figure 2 for the purification of a preparation of [U-^{14}C]ANAP on a column of DEAE-Sephadex A-25. The compound was eluted at 0.3 M triethylammonium bicarbonate, pH 7.4. Iodination of ANAP cannot be achieved directly on ANAP with either iodogen or iodine monochloride. The best method is to iodinate 4-azido-2-nitrophenol with iodine monochloride at an alkaline pH. In this way, radioactive iodine can be used to systhesize radioactive ANAP.

Photochemical properties of ANAP. The absorption spectrum of ANAP

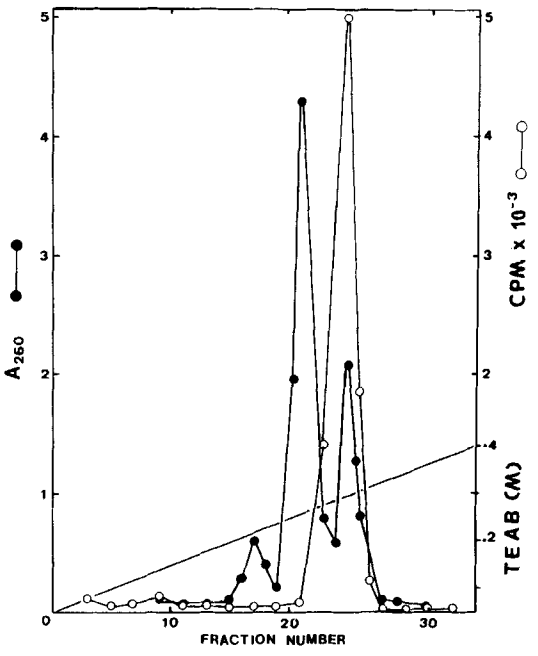

Figure 2. Purification of radioactive ANAP on a DEAE-Sephadex column. Crude [U-^{14}C]ANAP preparation was applied onto a (1 x 25 cm) column of DEAE-Sephadex A-25-120 and eluted with a 200 ml gradient of 0.01 to 0.4 M triethylammonium bicarbonate (TEAB), pH 7.4.

is characteristic of the adenosine moiety and the light sensitive azidophenyl group. As shown in Figure 3, ANAP has an absorption maximum at 250 nm and a broad band at around 320 nm. The former arises from the adenine and the phenyl rings, and the latter from the nitrophenyl moiety. On photolysis, the peak at 250 nm decreases as well as red-shifts with a concomitant increase at the 320 nm region. The kinetics of photolysis is complex. Only the initial reaction is a first order process which was estimated to have a half-life of about 1 min under the conditions indicated in Figure 3. The highly reactive nitrene generated on irradiation can undergo several reactions, such as insertion into C-H and N-H bonds, hydrogen abstraction from surrounding molecules, addition to double bonds, condensation and rearrangement [13]. These

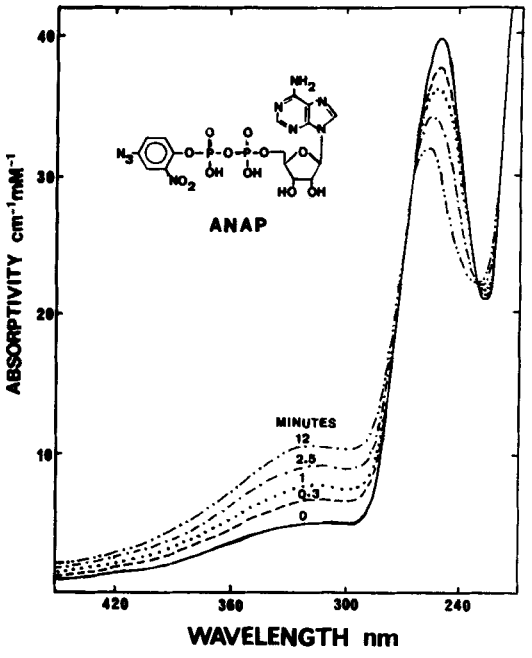

Figure 3. Absorption spectra of ANAP under photolysis. A solution of ANAP in 50 mM potassium phosphate, pH 7.0, was irradiated through a one-eighth inch-think glass filter with a Blak-Ray Longwave ultraviolet lamp at a distance of 10 cm. The spectra were taken after the indicated time of irradiation. Photolysis can also be achieved with hand-held Model UVS-11 mineralight lamp.

series of complicated reactions can occur both intra- and intermolecularly and probably account for the observed kinetics.

Enzyme inhibition. Figure 4 shows that ANAP is a competitive inhibitor of glucose-6-phosphate dehydrogenase against NADP with an inhibition constant of 9.5 μM. The apparent Km for NADP determined from Figure 4 is 35 μM, which is in good agreement with that of 56 μM reported in literature [14]. Thus ANAP binds at least three fold better than the coenzyme. This high affinity of ANAP is also reflected by the photoinactivation of the enzyme which can be achieved with Model UVS-11 mineralight lamp. In the presence of 78 μM ANAP, the enzyme is inactivated 81% on irradiation. When radiolabeled ANAP was used, radioactivity was also incorporated into the protein. At high concentrations of

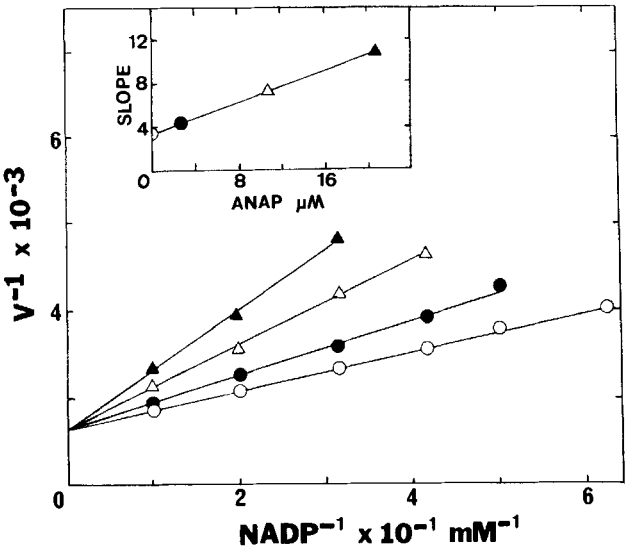

Figure 4. Competitive inhibition of ANAP against NADP. Glucose-6-phosphate dehydrogenase was assayed using various concentrations of NADP at fixed ANAP levels. The ANAP concentrations employed are: o——o , 0 μM; ●——● , 3.1 μM; △——△ , 11.4 μM; ▲——▲ , 21.3 μM. Insert: Secondary plot of slope versus ANAP concentration.

NADP, the enzyme can be protected from such photoinactivation indicating that ANAP inactivates the enzyme by binding to its active site.

Iodination of ANAP does not affect its binding to the enzyme. An inhibition constant of 6.6 μM was obtained through a similar analysis of inhibition kinetics.

Thus ANAP is a good analogue of NADP, at least in the case of glucose-6-phosphate dehydrogenase. It is possible that this compound may also serve as a good analogue of NAD for other enzyme systems. In our studies with cAMP-dependent protein kinase, ANAP also competitively inhibits the enzyme against ATP and consumes its activity in a light-dependent manner (to be published). Therefore ANAP may serve as a general analogue for adenosine nucleotides. This compound may be useful in studies of structures of adenosine nucleotide binding proteins.

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