Synthesis and Photoaffinity Labeling of 3'(2')-O-(p-azidobenzoyl) ATP

Seungjin Shin, Wookyoung Lee and Jong-sang Park*

Center for Molecular Catalysis, Department of Chemistry, Seoul National University, Seoul 151-742, Korea
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Abstract : A photoactive analog of ATP, 3'(2')-O-(p-azidobenzoyl)-adenosine 5-triphosphate (AB-ATP) was synthesized by chemically coupling N-hydroxysuccinimidyl-4-azidobenzoate (NHS-AB) and ATP. The utility of AB-ATP as an effective active-site-directed photoprobe was demonstrated using catalytic subunit of protein kinase A as a model enzyme. Photoincorporation of AB-ATP was saturated with apparent dissociation constant of 30 μM and protected completely by 100 μM of ATP. When the enzyme was covalently modified by photolysis in the presence of saturating amounts of photoprobe, about 60% inhibition of enzyme activity was observed. These results demonstrate that AB-ATP has potential application as a probe to characterize ATP-binding proteins including protein kinases.

Key words : AB-ATP, photoaffinity labeling, protein kinase A, synthesis of ATP analogs

Various types of photoreactive analogs of ATP have been used to identify and study the ATP-utilizing enzyme. Of these, azido derivatives of ATP are currently used and contain photoreactive groups placed mostly in the 2- or 8- position of adenine ring. These analogs have proven to be very effective photoprobes for a number of ATP binding proteins (Wagenvoord et al., 1980 ;Potter et al., 1983; Zhou et al., 1988; Joseph et al., 1993) However, there are a number of ATP binding proteins that do not bind the 2- or 8-azido derivatives (Cross et al., 1987; King et al., 1989). The difference in binding may be caused by the syn-conformation of 8-azido ATP (Czarnecki et al., 1982) and tautomerization of 2-azido ATP (Joseph., 1984).

ATP analogs which are not modified at the adenine ring have also been widely used for identifying ATP-binding site of enzymes (Richard et al., 1975; Mahmood et al., 1984). However, these analogs have the shortcoming that photoactive groups are in distance. Thus, in order to localize the ATP binding site on ATP-utilizing enzymes clearly, several photoaffinity probes should be used.

AB-ATP, synthesized and used in this experiment, has unmodified adenine base and the arylazido group at a relatively short distance. The results reported herein indicate that AB-ATP will be useful for studying various aspects of ATP binding protein including protein kinases.

*To whom correspondence should be addressed.
Tel : 82-2-880-6660, Fax : 82-2-889-1568.
E-mail : pfjspark@plaza.snu.ac.kr

Materials and Methods

Materials

Catalytic subunit of protein kinase A was purchased from Sigma (St. Louis, USA) and radioactive nucleotides ([γ-^{32}P] ATP, 3000 Ci/ mmol) were purchased from Amersham Corp. (Amersham, UK). Other chemicals were purchased from Sigma or Aldrich (Milwaukee, USA). Nucleotide solutions were prepared freshly before use.

Synthesis of 3'(2')-O-(p-azidobenzoyl)-adenosine 5-triphosphate (AB-ATP)

NHS-AB (45.5 mg, 0.175 mmole) was dissolved in 0.2 ml of dimethylformamide followed by the addition of 0.2 ml of ATP (20.1 mg, 0.035 mmole) in 0.24 M triethylamine bicarbonate (TEAB) buffer, pH 7.4. The reaction was allowed to proceed for 4-8 hours at 30°C with mild stirring. The solvent was then removed by evaporation, and the residue was dissolved in 1 ml of 50 mM ammonium acetate buffer (pH 6.8) and centrifuged to separate precipitates containing excess NHS-AB and side products. AB-ATP was then purified by DEAE-cellulose chromatography. The solution was applied to a 10-ml DEAE-cellulose column and gradient-eluted with 200 ml of ammonium acetate buffer (A: 50 mM, B: 500 mM, pH 6.8) with 1.5 ml flow rate. Elution was monitored by the absorbance at 260 nm. Each peak was pooled, evaporated to dryness, and redissolved in water. Each peak was identified with TLC, UV absorption spectra and NMR spectra. The yield of AB-ATP routinely obtained was about 40% with respect to

the added ATP.

Enzyme assay

Activity of protein kinase A was assayed by the method of Roskoski (1992). Synthetic peptide residue, Kemptide (LRRASLGW), was synthesized by solid phase peptide synthesis.

The substrate concentrations used were 0.1 mM Kemptide and 1 mM [γ-^{32}P] ATP (400 cpm/ pmol) in 50 mM Tris (pH 7.5) and 20 mM $MgCl_2$. Reaction was started by adding enzyme and the reaction mixture (200 µl). The mixture was then filtered on the phosphocellulose paper and the radioactivity of the paper was counted in a liquid scintillation counter.

Photoaffinity labeling with AB-ATP

Photoaffinity labeling was initiated by the addition of 30 µM of [γ-^{32}P] AB-ATP to enzyme sample. Reaction buffer had no Kemptide and $MgCl_2$. After 1 min incubation of photoprobe at room temperature, the samples were photolyzed with UV irradiation (254 nm, 25 W/cm^2) for 1 min at a distance of 5 cm. The reaction was quenched by addition of 0.25 vol. of 4× sample buffer consisting of 250 mM Tris-HCl, pH 6.8, 8% SDS, 20% β-mercaptoethanol, 40% glycerol, and bromophenol blue (tracking dye). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the protein band was sliced and the radioactivity was measured.

Results

Synthesis of 3 (2)-O-(p-azidobenzoyl)-adenosine 5-triphosphate (AB-ATP)

The synthetic scheme of AB-ATP is shown in Fig. 1. As a result of nucleophilic substitution in H_2O, N-hydroxy succinimide and azidobenzoic acid were made and side products, including NHS-AB, were easily separated on anionic exchange chromatography for their nonionic characters (Fig. 2). Each peak was identified preliminary by TLC on silica using the n-butanol/water/acetic acid system (5:3:2, v/v/v) by comparison with standard molecules. Purified AB-ATP (R$_f$, 0.35) was completely hydrolyzed to azidobenzoic acid (R$_f$, 0.95) and ATP (R$_f$, 0.1) in the basic condition of 1 M NH$_4$-HCO$_3$, which showed the existence of base labile ester linkage.

To determine the precise structure of AB-ATP, about 5 mg of purified AB-ATP was dissolved in D_2O and analyzed with ^{1}H NMR. ^{1}H NMR spectrum of AB-ATP (Fig. 3) showed that δ 8.55 (1.05H, C$_8$, 3'-isomer), 8.45 (0.8H, C$_8$, 2'-isomer), 8.2-7.2 (4.7H, C$_2$+benzene rings), 6.4 (0.6H, C$_1$, 2'-isomer), 6.3 (0.9H, C$_1$, 3'-isomer), 5.7
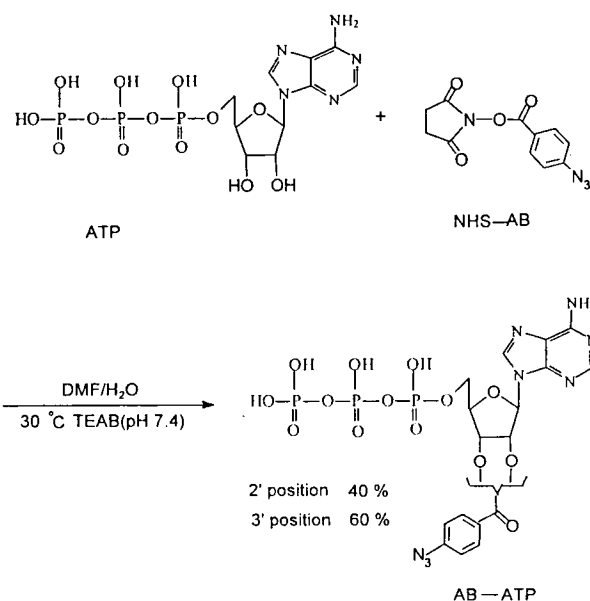

Fig. 1. Scheme of the synthesis AB-ATP. The synthetic method was described under "Materials and Methods". AB-ATP contained photoactive arylazido group at the ribose ring. By e-quilibration, AB-ATP exsits as a mixutre form of 2' and 3' isomer.
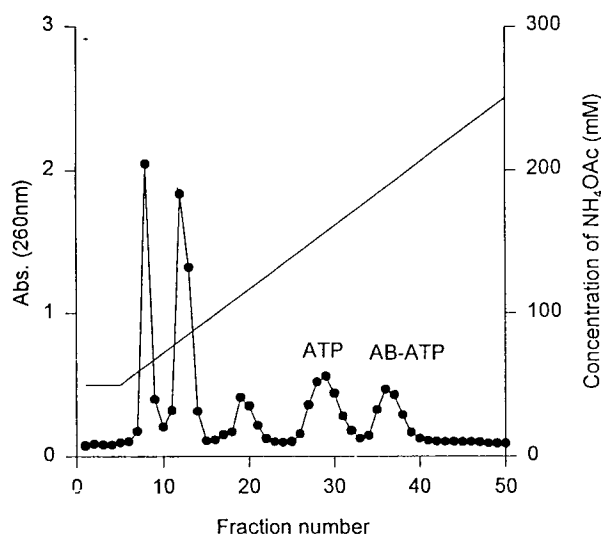

Fig. 2. Purification of AB-ATP. AB-ATP was purified by DEAE-cellulose column chromatography. Each peak was monitored by optical density at 260 nm and identified by TLC, UV-absorption spectra and ^{1}H-NMR analysis. For different molecules, peak intensity can not be correlated with quantity. AB-ATP has hydrophobic arylazido group and this may be reason for the later elution than ATP

(1.9H, C$_2$, 2'-isomer+C$_3$, 3'-isomer), 5.1 (1.17H, C$_2$, 3'-isomer), 4.8-4.6 (0.87H, C$_3$, 2'-isomer and C$_4$, 3'-isomer were under the H_2O peak), 4.3 (0.7H, C$_4$, 2'-isomer), 4.15 (4H, C$_5$). This ^{1}H NMR spectrum indicates that AB-ATP is a mixture of 3'- and 2'-monosubstituted isomers in a ratio of 60:40. The total peak integration attrib-
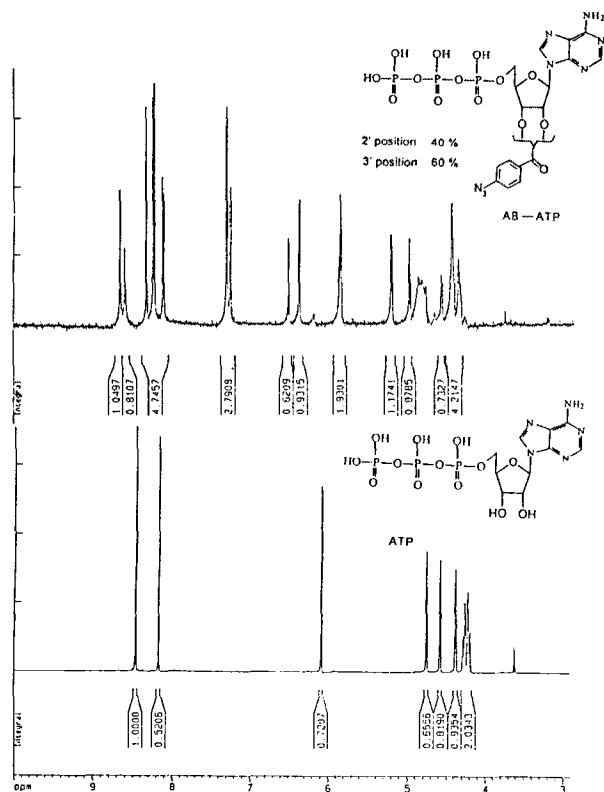
Fig. 3. ^{1}H-NMR analysis of AB-ATP. About 5 mg of purified AB-ATP was dissolved in D_2O. and analyzed by 500 MHz ^{1}H-NMR 11.7 Tesla in Inter-University Center of Natural Science Research Facilities, Seoul National University. Compared with the NMR spectrum of ATP, AB-ATP showed the mixture forms of 2' and 3' isomers.

uted to the azidobenzoyl group protons and the adenosine protons was consistent with one azidobenzoyl group/adenosine and ruled out the presence of a possible 2,3-disubstituted compound.

Photoaffiinity labeling of protein kinase A

Catalytic subunit of Protein kinase A was photolabeled with [γ-^{32}P] AB-ATP. As shown in Fig. 4, the enzyme was not autophosphorylated with [γ-^{32}P] ATP (lane 1). Autophosphorylation, of course, is a typical phenomenon in protein kinases (Jean et al., 1992), but it was negligible in the photolabeling condition which employs deletion of Mg^{2+} ion and short incubation time. Enzyme was labeled with [γ-^{32}P] AB-ATP under UV irradiation and was not labeled under dim light (lane 2, 3). It means that radiolabeling of the enzyme results from U-V-induced labeling. With the increase of concentration of nonradiolabeled ATP, labeling was disappeared (lane 4, 5). These results suggest that [γ-^{32}P] AB-ATP labels the active site of the enzyme.

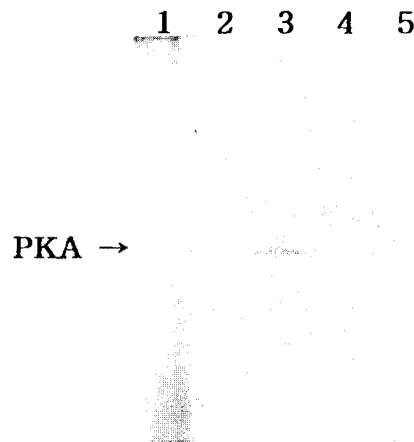To demonstrate saturation effects with [γ-^{32}P] AB-ATP, the enzyme was photolabeled with increasing con-

Fig. 4. Autoradiography of photoaffinity labeling of protein kinase A by [γ-^{32}P] AB-ATP. [γ-^{32}P] AB-ATP (30 μM) was incubated for 1 min and photolyzed for 1 min with UV-light (254 nm, 25 W/cm^2) at a distance of 5 cm. The photolabeled proteins were analyzed by SDS-PAGE and the gel was stained, dried, and autoradiographed. Enzyme was incubated with 100 μM of [γ-^{32}P] ATP (lane 1) and [γ-^{32}P] AB-ATP without UV light (lane 2). Photoaffinity labeling with [γ-^{32}P] AB-ATP under UV light was done (lane 3, AB-ATP only) in the presence of 5 μM ATP (lane 4) or 50 μM ATP (lane 5).
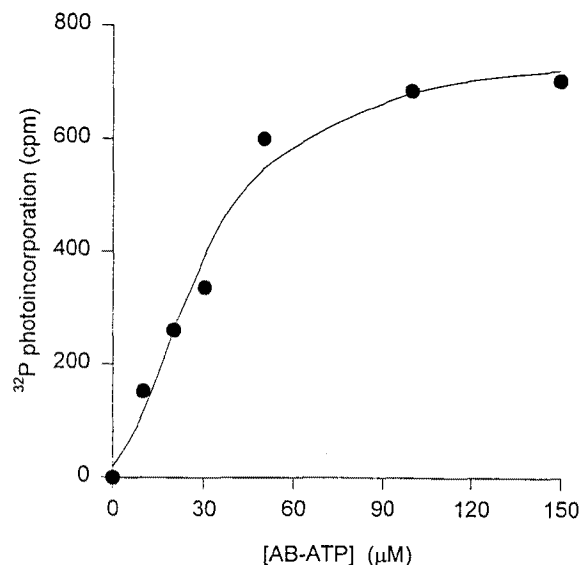

Fig. 5. Saturation of [γ-^{32}P] AB-ATP photoincorporation into protein kinase A. Enzymes were photolabeled with increasing concentration of [γ-^{32}P] AB-ATP. Incorporation of [γ-^{32}P] AB-ATP was quantitated by excising the labeled protein band from the gel and determining its radioactivity by liquid scintillation counting.

centration of [γ-^{32}P] AB-ATP. The apparent value for the dissociation constant (K_d) was found to be 30 μM. The data in Fig. 5 demonstrate the saturability of ATP-specific binding site of protein kinase A with this photo-probe and thereby decreases the possibility of nonspecific labeling.
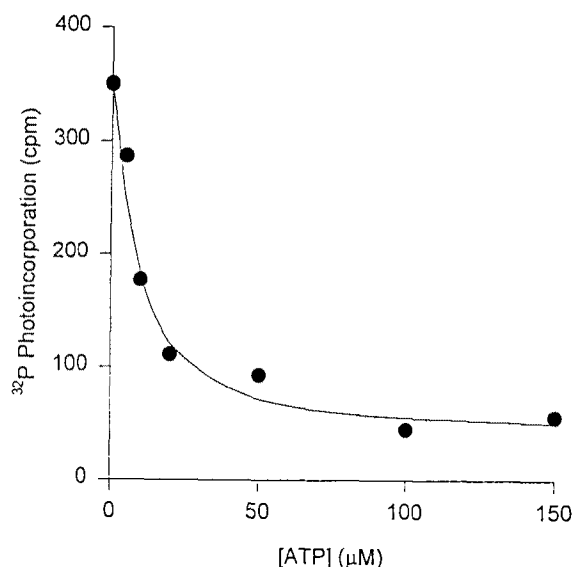
Fig. 6. Protection against photolabeling by [γ-^{32}P] AB-ATP in the presence of an increasing concentration of ATP. The concentration of [γ-^{32}P] AB-ATP used was 30 μM.

To further demonstrate photolabeling of AB-ATP at the active site, the enzyme was photolyzed with 30 μM [γ-^{32}P] AB-ATP in the presence of ATP (Fig. 6). About 90% of the photolabeling was protected by 100 μM ATP and the half-maximal protection was observed at approximately 10 μM ATP.

Photoinactivation of the protein kinase A

The ability of AB-ATP to inhibit protein kinase A activity by photolabeling upon irradiation under different condition was investigated. As shown in Table 1, when the enzyme was photolyzed in the presence of 50 μM AB-ATP and assayed subsequently, about 60% activity was lost. Photolysis in the absence of probe affected slightly the enzyme activity. The inactivation by the probe could be protected by photolyzing in the presence 100 μM ATP. These results indicate that the analog was photoinsertioning into ATP binding site on the enzyme in a specific manner.

Discussion

3'(2')-O-(p-azidobezoyl) ATP (AB-ATP) was synthesized by esterification of ATP and NHS-AB in a simple one-step reaction. The esterification can occur in 2'- or 3'-OH group, but 2'-OH group may be kinetically more reactive for substitution than 3'-OH. This can explain the fact that when the 2'-deoxy ATP was instead used, no azidobenzoyl ATP was made (data not shown). However, such a substitution is relatively less stable in comparison to the thermally favorable esterification of

Table 1. Photoinactivation of protein kinase A by AB-ATP

Condition	hν	Activity
		%
No addition (Control)	-	100
No addition	+	89
50 μM AB-ATP	-	92
100 μM ATP	+	87
50 μM AB-ATP	+	43
50 μM AB-ATP+100 μM ATP	+	78

A volume of 100 μl of enzyme (1 μg) was photolabeled in 50 mM Tris (pH. 7.5) containing 50 μM of AB-ATP. An aliquot (10 μl) was taken and assayed as described under "Materials and Methods" .

the 3'-hydroxyl group, thus the 2' isomer can be converted to the 3' isomer by acyl migration and the products are in the equilibration state. This conversion is well supported by a number of studies (Zamecnik et al., 1962; McLaughlin et al., 1965). Thus the synthesized AB-ATP may be a mixture forms of 2' and 3' isomer, which was identified by the NMR analysis in our experiment.

Many of arylazido ATP can be found in the writings of Guillory and Jeng (1977). They described a great deal of the chemistry about the esterification of the ribose hydroxyl group of ATP and showed the much use of arylazido-β-alanyl ATP in biological system. However, contrary to the much use of arylazido-β-alanyl ATP, the utilization of AB-ATP, used in this experiments, has rarely been reported. Thus the newly synthesized arylazido ATP, AB-ATP needs to be studied about its properties.

To test the efficacy of synthesized analog, protein kinase A was chosen for the initial photoaffinity labeling experiments. The photoaffinity probe, AB-ATP proved to have all the proper characteristics of a useful nucleotide photoaffinity probe for investigating ATP binding protein. Both the saturation effects of [γ-^{32}P] AB-ATP as a photoaffinity probe and prevention of photoinsertion by ATP clearly demonstrate that the photoprobe interacts with active site of the enzyme. This is also supported by the 60% inhibition of the enzyme activity by the photolysis with a saturating amount of AB-ATP and the recovery of its activity in the presence of ATP.

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