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# 3'-ARYLAZIDO-8-AZIDO ATP - A CROSS-LINKING PHOTOAFFINITY LABEL FOR ATP BINDING PROTEINS

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SUMMARY: The synthesis of 3'-O-{3-[N-(4-azido-2-nitrophenyl) amino] propionyl} 8-azido-adenosine 5'-triphosphate - a 3'-arylazido-8-azido ATP - is described. The ATP derivative is characterized by thin layer chromatography, infrared spectroscopy, and optical spectroscopy. Its photolysis upon irradiation with uv light and its stability in dependence on pH are tested. Its two photolabile azido groups allow the use of this ATP analog as a photoaffinity label for cross-linking the subunits of ATP binding proteins.

## INTRODUCTION

Two types of ATP analogs have been successfully used in photoaffinity labeling of nucleotide binding sites of various proteins - 3'-arylazido ATP analogs (1-9) and 8-azido ATP analogs (10-19). For example Lunardi et al. (3), Cosson and Guillory (6), and Carlier et al. (7) have labeled the $F_1$ ATPase from beef heart mitochondria and chloroplasts, respectively, with 3'-arylazido nucleotides. Wagenvoord et al. (14) have successfully labeled the ß subunit of mitochondrial $F_1$ ATPase with 8-azido ATP. Scheurich et al. (15) and Schäfer et al. (19) have preferentially labeled the ß subunit of bacterial $F_1$ ATPase with 8-azido ATP and with the fluorescent 8-azido-1,$N^6$-etheno ATP. All these ATP analogs are hydrolyzed by the $F_1$ ATPase to the corresponding diphosphates.

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In order to develop a photoaffinity label capable of cross-linking protein subunits we have introduced both the photolabile azido and arylazido group into the same molecule.

## MATERIALS AND METHODS

4-Fluoro-3-nitroaniline was purchased from Ega-Chemie. Carbodiimidazol was purchased from Sigma Chemical Co. ATP disodium salt and all other chemicals were obtained from E. Merck. 4-Fluoro-3-nitrophenyl azide and N-4-azido-2-nitrophenyl-ß-alanine were synthesized as described by Jeng and Guillory (1). 8-Azido ATP was synthesized as described by Schäfer et al. (20).

3'-O-{3-[N-(4-azido-2-nitrophenyl) amino] propionyl} 8-azido-adenosine 5'-triphosphate (3'-arylazido-8-azido ATP):

N-4-Azido-2-nitrophenyl-ß-alanine (63 mg, 0.25 mmole) and carbodiimidazol (48.5 mg, 0.3 mmole) were dissolved in 200 µl of freshly distilled dimethylformamide and stirred for 15 min in the dark at room temperature. Then a solution of 8-azido ATP triethylammonium salt (42.5 mg, 0.05 mmole) in 1.0 ml of water was added. The reaction mixture was stirred in the dark for 7 hrs at room temperature. Then the solvent was evaporated in the vacuum. The residue was washed repeatedly with dry acetone, centrifuged, and dried in the vacuum. Then it was redissolved in 50-100 µl of water and centrifuged. The clear solution was applied to descending paper chromatography on a sheet (18x40 cm) of paper (type 2040 B, Schleicher und Schüll) and eluted with n-butanol/water/acetic acid (5:3:2 v/v). The broad orange band ($R_f$ = 0.4) was eluted with water and lyophilized. The residue was stored at -20 °C in the dark. Yield: 3.5 % (spectroscopically).

Thin layer chromatography was carried out on cellulose plates (CEL 300-10, Macherey-Nagel und Co.) and developed in either solvent system A (isobutyric acid/water/ammonia 66:33:1 v/v), or solvent system B (n-butanol/water/acetic acid 5:3:2 v/v). The infrared spectrum was recorded with a Perkin-Elmer 735 B infrared spectrophotometer. The optical absorption spectra were taken with a Cary 118 spectrophotometer. The photolysis was followed spectroscopically. Irradiation was performed with a Mineralight handlamp UVSL 25 at position 'long wave'. The distance between the light source and the sample was 4 cm. The energy fluence rate at the sample was $4 \times 10^3$ $W \cdot m^{-2}$. The sample was irradiated at 20 °C. The irradiation time between two following uv recordings was 30 sec initially. It increased up to 30 min towards the end of the photolysis. A final spectrum was taken after 90 min of irradiation time.

Photoaffinity labeling of $F_1$ ATPase from Micrococcus luteus was carried out as described earlier (15,19).

## RESULTS AND DISSCUSSION

In order to produce a bifunctional photolabile ATP analog - suitable for photoaffinity cross-linking of protein subunits - we have esterified N-4-azido-2-nitrophenyl-ß-alanine with

8-azido-adenosine 5'-triphosphate (Fig. 1). This synthesis was conducted according to that of 3'-arylazido ATP analogs as described by Jeng and Guillory (1).

Descending paper chromatography revealed two major orange bands ($R_f$= 0.4 and $R_f$= 0.9) and a colorless uv absorbing band ($R_f$= 0.1). The orange band ($R_f$= 0.4) contains 3'-arylazido-8-azido ATP. The orange band ($R_f$= 0.9) and the colorless band ($R_f$= 0.1) were identified spectroscopically as N-4-azido-2-nitrophenyl-ß-alanine and 8-azido ATP, respectively. The product obtained after paper chromatography ($R_f$= 0.4) and freeze drying is homogeneous according to thin layer chromatography on cellulose plates. Only a single orange-red spot is observed in the two different solvent systems A and B.

The infrared spectrum of 3'-arylazido-8-azido ATP shows two bands at 2130 $cm^{-1}$ and 2170 $cm^{-1}$ which are characteristic for $N_3$ stretching vibrations (21). This result indicates the presence of two different azido groups. The position of the band at 2170 $cm^{-1}$ is identical with the position of the infrared band

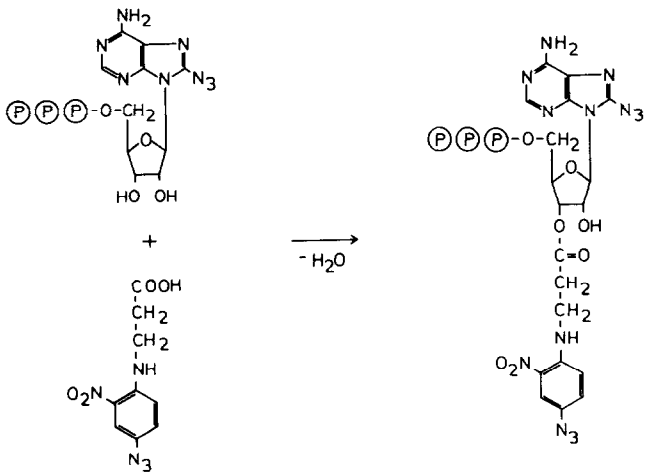


Fig. 1 Synthesis of 3'-arylazido-8-azido ATP

($N_3$ stretching vibration) of 8-azido ATP (20). The optical absorption spectrum shows two maxima at 475 nm and 263 nm (Fig. 2, spectrum C). The absorption is quite similar to that of 3'-arylazido ATP (1).

The photochemical activity of the compound is demonstrated by its ability to bind irreversibly to cellulose on thin layer plates after exposure to uv light prior to the contact with the elution solvent. A colored spot remains at the origin after development of the chromatogram in contrast to the not irradiated control. The photolysis of 3'-arylazido-8-azido ATP in aqueous

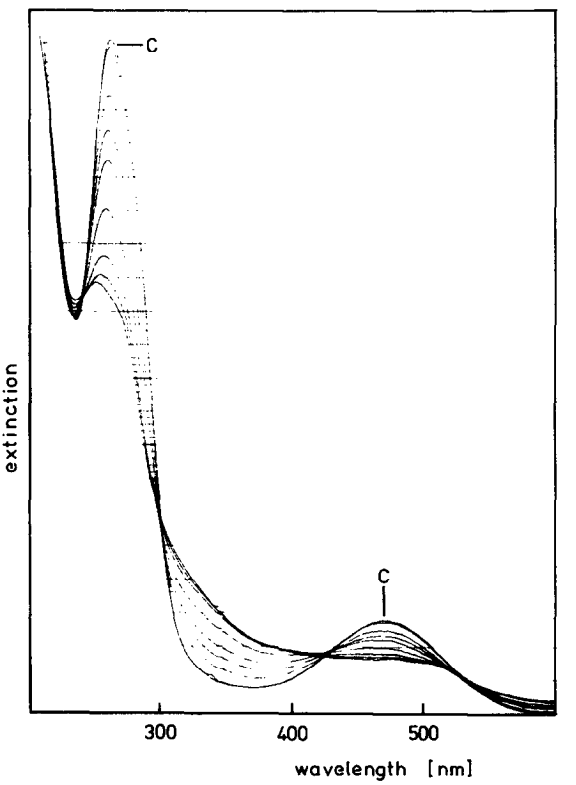


Fig. 2 Change of the optical absorption spectrum of 3'-arylazido-8-azido ATP upon irradiation with uv light in aqueous solution (pH= 6.1). The sample was irradiated at 20 °C. The irradiation time between two subsequent recordings was from 30 sec (initially) to 30 min (towards the end of the photolysis). The final spectrum was taken after 90 min of irradiation time. (C = control without irradiation).

solution was followed spectroscopically (Fig. 2). The compound is quickly decomposed by irradiation with uv light. The maxima at 475 nm and 263 nm decrease and the maximum at 263 nm shifts to 250 nm. The occurrence of the isosbestic points suggests that the photolysis is largely controlled by a single set of reactions.

3'-Arylazido-8-azido ATP is quite unstable at neutral or alkaline pH. Incubation of the sample at these pH values results in the hydrolysis of the ester linkage to N-4-azido-2-nitrophenyl-ß-alanine and 8-azido ATP. Jeng and Guillory (1) have observed a similar behaviour of 3'-arylazido ATP. In addition to the hydrolysis the formation of an insoluble (in water, or acetone) red material takes place upon longer (3 days) storage of 3'-arylazido-8-azido ATP (probably polymerisation products of the bifunctional compound). For these reasons it is recommended to use freshly synthesized samples for all experiments.

The double photoaffinity label can be applied to elucidate how close the nucleotide binding site is localized to a neighboring subunit in an oligomer protein. Cross-linking should occur when the distance between the binding site towards the next subunit is maximal about 20 Å. Cross-linking should be most effective when the binding site is localized at the interphase between two subunits. The photoaffinity label therefore might prove to be a useful tool for an approach to these questions which are of great interest concerning the possible interaction of binding sites with neighboring subunits and the putative consequences for half the site's reactivity.

Preliminary results indicate that 3'-arylazido-8-azido ATP is an effective cross-linking photoaffinity label for $F_1$ ATPase from Micrococcus luteus (22). The enzyme hydrolyzes this ATP

analog. Irradiation of the enzyme with uv light in the presence of the label and $Mg^{2+}$ ions leads to a drastic reduction of its activity. This inactivation can be prevented by addition of ADP, or ATP, and $Mg^{2+}$ ions prior to the illumination procedure. Sodium dodecyl sulfate gel electrophoresis of the labeled enzyme reveals an additional protein band with a molecular weight of 120 000 (α subunit: 65 000; ß subunit: 55 000). This result suggests the formation of cross-links between two of the major subunits, probably between one α and one ß subunit. The composition of this cross-linked product is presently under investigation.

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