3′-Arylazido-β-alanyl-2-azido ATP, a Cross-Linking Photoaffinity Label for F_1ATPases

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The synthesis of the 3′-arylazido-2-azido ATP derivative 3′-O-{3-[N-(4-azido-2-nitrophenyl)-amino]propionyl}2-azido-adenosine 5′-triphosphate (2,3′-DiN_3ATP) is described. The bifunctional photoreactive ATP analog is characterized spectroscopically. Photoaffinity labeling of F_1ATPase from *Micrococcus luteus* by this analog results in the inactivation of the enzyme and in the formation of higher molecular weight cross-links, composed of α- and β-subunits.

Introduction

Photoaffinity labeling [1, 2] and photoaffinity cross-linking [3] of various F_1ATPases by photoactivatable nucleotide analogs have demonstrated the position of the catalytic nucleotide binding sites at the β-subunit to be in the direct proximity of an α-subunit – probably at the interface of these subunits. This conclusion bases mainly on the nucleotide specific formation of α-β cross-links upon photoaffinity cross-linking with the photoreactive bifunctional 3′-arylazido-β-alanyl-8-azido ATP (8,3′-DiN_3ATP) [4, 5]. In contrast to ATP the 8-azidoadenine nucleotides are preferentially in *syn* conformation due to the azido group in position 8 of the adenine ring [6]. This property could hamper an efficient binding of 8,3′-DiN_3ATP to F_1ATPases. For this reason we have synthesized the 2-azido ATP analog 2,3′-DiN_3ATP which, like ATP, is expected to favor the *anti* conformation [7].

Materials and Methods

4-Fluoro-3-nitroaniline was purchased from Ega-Chemie. 1,1′-Carbonyldiimidazole and 2-chloroadenosine were obtained from Sigma Chemical Co. All other chemicals were of highest purity available from commercial sources. 4-Fluoro-3-nitrophenyl azide and N-4-azido-2-nitrophenyl-β-alanine were synthesized as described by Jeng and Guillory [8]. 2-Azidoadenosine 5′-triphosphate was synthesized as described by van Dongen *et al.* [9] according to Schaeffer and Thomas [10], Sowa and Ouchi [11], and Hoard and Ott [12].

3′-O-{3-[N-(4-azido-2-nitrophenyl)amino]-propionyl}2-azidoadenosine 5′-triphosphate (2,3′-DiN_3ATP)

N-4-azido-2-nitrophenyl-β-alanine (63 mg, 0.25 mmol) and 1,1′-carbonyldiimidazole (48.5 mg, 0.3 mmol) were dissolved in 200 µl freshly distilled dimethylformamide. The solution was stirred in the dark at room temperature for 15 min. Then 2-azidoadenosine 5′-triphosphate triethylammonium salt (42.5 mg, 0.05 mmol) in 1 ml of water was added. The reaction mixture was stirred for 7 h in the dark at room temperature. After evaporation of the solvent under vacuum, the residue was washed repeatedly with dry acetone (until the supernatant became pale yellow), centrifuged and dried under vacuum. Then it was redissolved in a minimum amount of water and centrifuged. The products in the supernatant were purified by descending paper chromatography (paper: Type 2043 b, 18 × 40 cm, Schleicher und Schüll). The chromatogram was developed with *n*-butanol/water/acetic acid (5:3:2 v/v). The orange

Abbreviations: UV, ultraviolet; F_1, F_1ATPase from *Micrococcus luteus*; 8-N_3ATP, 8-azidoadenosine 5′-triphosphate; 2,3′-DiN_3ATP, 3′-O-{3-[N-(4-azido-2-nitrophenyl)-amino]propionyl}2-azidoadenosine 5′-triphosphate; 8,3′-DiN_3ATP, 3′-O-{3-[N-(4-azido-2-nitrophenyl)amino]-propionyl}8-azidoadenosine 5′-triphosphate.

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band near the front ($R_f = 0.9$) and the colourless band near the origin ($R_f = 0.1$) could be identified as the starting materials N-4-azido-2-nitrophenyl-β-alanine and 2-azido ATP, respectively. The broad orange region in the center of the chromatogram ($R_f = 0.45$) contains the bifunctional 2,3′-DiN_3ATP. It was eluted with water. 2,3′-DiN_3ATP was obtained by lyophilization and stored at −20 °C in the dark. Yield: 20% (spectroscopically). UV: $\lambda_{max} = 264$ nm, 475 nm.

The optical absorption spectra were recorded with a Cary 118 spectrophotometer. Photolysis of 2,3′-DiN_3ATP was followed spectroscopically. Irradiation was performed with a Zeiss LX 501 spectrophotometer ($\lambda = 310$ nm). The fluence rate at the position of the sample was 5 J/m^2·s. Absorption spectra were taken after 0, 2, 5, 10, 20, 40, and 60 min of irradiation time.

Preparation of F_1ATPase from *Micrococcus luteus* and determination of ATPase activity were performed as described before [4]. Photoaffinity cross-linking (Zeiss LX 501 spectrophotometer, $\lambda = 310$ nm) and hydrolytic cleavage of the formed cross-links were carried out analogous to [3, 4].

Results and Discussion

The esterification of N-4-azido-2-nitrophenyl-β-alanine with 2-azidoadenosine 5′-triphosphate results in the formation of the bifunctional photoactivatable 2,3′-DiN_3ATP (Fig. 1). The absorption spectrum of 2,3′-DiN_3ATP (Fig. 2, spectrum 0) resembles the absorption spectra of the corresponding ATP and 8-N_3ATP derivatives with maxima at 260 nm/480 nm, and 263 nm/475 nm, respectively [13, 14]. Photolysis of 2,3′-DiN_3ATP at 310 nm in aqueous solution results in the decomposition of the analog (Fig. 2).

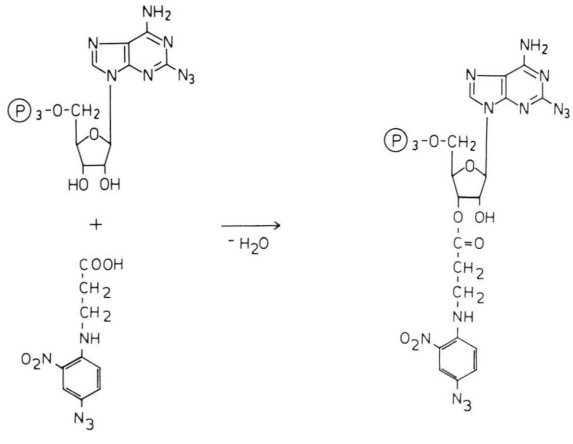

Fig. 1. Synthesis of 2,3′-DiN_3ATP.

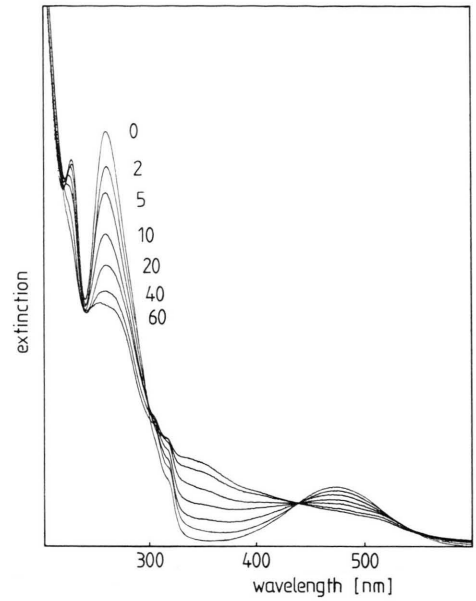

Fig. 2. Change of the optical absorption spectrum of 2,3′-DiN_3ATP (aqueous solution) upon irradiation with UV light (310 nm) at 20 °C. Irradiation time: 2, 5, 10, 20, 40 and 60 min (0 = unirradiated control).

Bifunctional photoreactive nucleotide analogs like 2,3′-DiN_3ATP can be applied to investigate the spatial arrangement of subunits in the multisubunit enzyme F_1ATPase by photoaffinity cross-linking [3]. This enzyme complex usually has the subunit stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. F_1ATPase from *Micrococcus luteus*, however, sometimes lacks the ε-subunit [15]. Six, probably three catalytic and three non-catalytic, nucleotide binding sites are distributed on the major subunits α and β as demonstrated by binding studies, affinity labeling, and photoaffinity labeling with nucleotide analogs [1, 16, 17]. Photoaffinity cross-linking should be advantageous for studying the neighborhood of these specific nucleotide binding sites. The nucleotide-dependent formation of cross-links can occur whenever the ligand's binding site at one subunit is adjacent to a second subunit, especially if it is localized at the interface of the two subunits.

The specific interaction of 2,3′-DiN_3ATP with F_1ATPase from *Micrococcus luteus* could be demon-

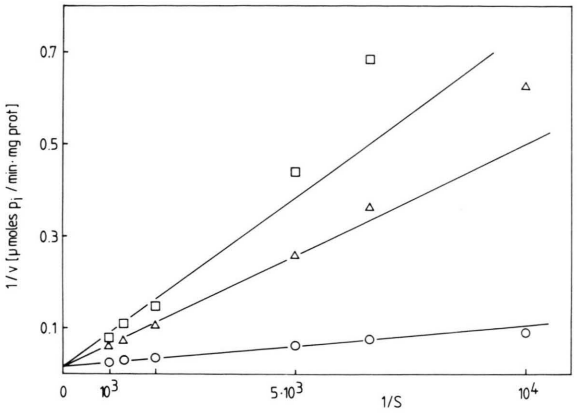

Fig. 3. The effect of 2,3′-DiN_3ATP on the hydrolysis of ATP. Plots of $1/v$ *vs.* 1/[Ca·ATP] of F_1ATPase from *Micrococcus luteus* in the absence of 2,3′-DiN_3ATP (○) and in the presence of 2,3′-DiN_3ATP [5 μM (△); 10 μM (□)]. The ATPase activity was determined at 37 °C in 5 ml test solution containing 1 μg F_1, 100 mM Tris-HCl (pH 8.0), different concentrations of 2,3′-DiN_3ATP (5 μM, 10 μM) and Ca·ATP ($[Ca^{2+}]/[ATP] = 5/1$). Prior to the addition of Ca^{2+} and ATP the test solution was incubated at 37 °C for 1 h.

strated by the competition of the analog with ATP for the catalytic site of the enzyme (Fig. 3). A competitive inhibition, however, was only observed at higher ATP concentrations ($[ATP] > 2\cdot10^{-4}$ M) [18]. UV irradiation of the enzyme in the presence of 2,3′-DiN_3-ATP and Mg^{2+} ions results in a drastic inactivation of the ATPase (Fig. 4). This inactivation could be observed neither by incubation of F_1ATPase with 2,3′-DiN_3ATP in the dark (dark control) nor by irradiation of the enzyme in the absence of the photoaffinity label (light control). The light induced inactivation coincides with the formation of a higher molecular weight cross-link ($m > 100$ kDa) (Fig. 5). Its electrophoretic mobility on SDS polyacrylamide gels and the SDS electrophoretic analysis of the cross-link's hydrolytic cleavage products indicate an α-β composition. The structure of the α-β cross-link is most likely analoguous to that of the α-β cross-links obtained by photoaffinity labeling of bacterial F_1ATPases with 8,3′-DiN_3ATP [4, 5]. The nucleotide specificity of the photoinactivation as well as of the cross-link formation could be proven by competition

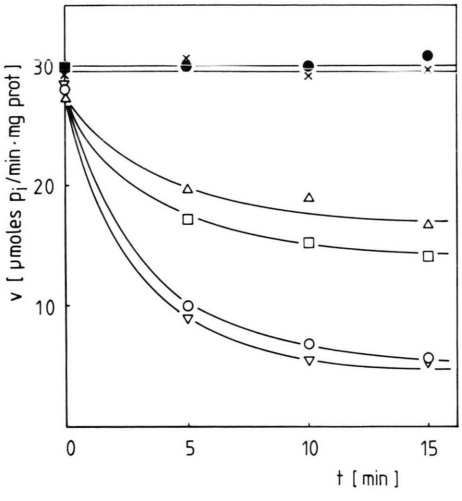

Fig. 4. Light-induced inhibition of F_1ATPase from *Micrococcus luteus*. Irradiation of F_1 in the presence of 0.02 mM Mg·2,3′-DiN_3ATP (○), light control in the absence of 2,3′-DiN_3ATP (×), dark control in the presence of 0.02 mM Mg·2,3′-DiN_3-ATP (●); irradiation of F_1 in the presence of 0.02 mM Mg·2,3′-DiN_3ATP and 1 mM Mg·AMP (▽), 1 mM Mg·ADP (△), or 1 mM Mg·ATP (□), respectively. The enzymatic activity was determined in 5 ml test solution containing 1 μg F_1, 100 mM Tris-HCl (pH 8.0), 5 mM Ca^{2+} and 1 mM ATP. Prior to the addition of Ca^{2+} and ATP the test solution was incubated at 37 °C for 1 h.

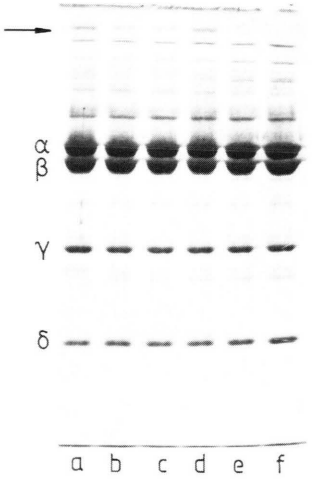

Fig. 5. Photoaffinity cross-linking of F_1ATPase from *Micrococcus luteus*. SDS electrophoresis gels of labeled (cross-linked) F_1ATPase:

a. F_1 labeled by 0.02 mM Mg·2,3′-DiN_3ATP
b.–d. F_1 labeled by 0.02 mM Mg·2,3′-DiN_3ATP in the presence of 1 mM Mg·ATP (b), 1 mM Mg·ADP (c), or 1 mM Mg·AMP (d)
e. F_1 irradiated in the absence of 2,3′-DiN_3ATP (light control)
f. native F_1.

experiments. Addition of the natural ligands ATP or ADP protects the enzyme partially from the attack of the bifunctional photoaffinity label as indicated by the reduction of the photoinactivation (Fig. 4) and of the cross-link formation (Fig. 5).

The low amount of cross-links formed (<5%) compared with the high inactivation of the enzyme (>80%) appears to be contradictory but a simple explanation is at hand. The major part of the bifunctional nucleotide is bound covalently to a catalytic site by only one photoreactive group and thus inactivates the enzyme. The second (photoactivated) azido group has a higher chance to react with water than with the enzyme as long as it is in an improper position. Also a second explanation may be considered. It is possible that the distance between the catalytic site at one subunit and the second subunit changes during the enzymic reaction. This distance may be suitable for cross-linking only during a certain phase of the reaction cycle.

Compared with 8,3′-DiN_3ATP the 2-azidoadenosine analog 2,3′DiN_3ATP is expected to be preferentially in the favorable anti conformation as the natural ligands of F_1ATPases [6]. Nevertheless, two disadvantages of 2,3′-DiN_3ATP have to be mentioned. Firstly, photoaffinity labeling with 2-azidoadenine nucleotides is complicated by the tautomerism of these analogs [7]. In neutral aqueous solution, for example, only 45% of 2-azido ATP is found to be the photoreactive azido isomer. The major portion of this photoaffinity label is present in form of two non-photoreactive tetrazolo derivatives. The equilibrium depends on the pH value as well as on the polarity of the solvent. Secondly, these results are more ambigous due to the increased maximal distance between the photoreactive groups of 2,3′-DiN_3ATP. In comparison to 8,3′-DiN_3ATP (maximal distance between the azido groups: ~2 nm) both azido groups of 2,3′-DiN_3ATP are separated additionally by about 0.2 nm. A specific cross-linking between amino acid residues of the nucleotide binding sites and those of the adjacent subunits should occur up to this distance (~2.2 nm).

Our results demonstrate the suitability of 2,3′-DiN_3ATP for photoaffinity cross-linking of F_1ATPase from *Micrococcus luteus*. They support our hypothesis concerning the localization of the catalytic nucleotide binding sites on the β-subunits in direct proximity to the α-subunits [4, 14].

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