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Synthesis of a photoaffinity-spin-labeled derivative of ATP and its first application to F_1-ATPase

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The synthesis of an ATP derivative is described, in which a spin-label is attached to the 3′-position of the ribose moiety and an azido group to C8 of the adenine ring (SL-N_3-ATP). Irradiation of this compound at 350 nm generates a nitrene, which will react with any functional group in its vicinity. SL-N_3-ATP exhibits a strongly immobilized ESR spectrum in a complex with F_1-ATPase from beef heart mitochondria. It was covalently incorporated into this enzyme. SL-N_3-ATP may be employed in ESR investigations under conditions in which non-covalent interactions are too weak for motionally restricted species to be easily observed.

F_1-ATPase; Spin-labeled ATP; Photoaffinity-spin-labeled ATP

1. INTRODUCTION

Spin-labeled nucleotides have been employed in various studies of structure-function relationships of enzymes [1]. In the case of adenine nucleotides, the spin-label was either bonded to N^6 or C8 of the adenine moiety [2] or to the 3′-position of the ribose [3]. Besides simple binding studies, in which the residual signal amplitude of the freely tumbling spin-labeled nucleotide in the presence of the enzyme is used as a measure for complex formation, important information can be obtained from the lineshape of the motionally restricted, enzyme bound species. Changes in this lineshape can be correlated, e.g., with conformational changes in the vicinity of the label [4]. Moreover, distances between spin-labeled nucleotides bound to adjacent sites on the same macromolecule can be determined from dipolar splitting of the spectra as shown in the case of spin-labeled NAD^+ [5].

Such investigations are generally limited to conditions of tight binding, because the much narrower and stronger signal of the free component will otherwise dominate the spectrum. Photoaffinity derivatives of natural ligands allow for covalent and specific binding of the ligand to its receptor. We have recently introduced NAD^+ analogs that contain both a spin-label and a photoaffinity group [6] and which eliminate therefore the problems associated with freely tumbling components. Here we describe a photoaffinity-spin-label-ATP-derivative, SL-N_3-ATP (fig.1), and its first application to studies of mitochondrial F_1-ATPase.

F_1-ATPases from most species exhibit a $\alpha_3\beta_3\gamma\delta\epsilon$ subunit composition with up to six nucleotide-binding sites located on the α- and β-subunits. The so-called loose sites appear to play a role primarily in catalysis, whereas the tight sites are filled in most enzyme preparations with slowly exchanging ADP [7–9]. These tightly bound nucleotides can, however, be removed in the case of the mitochondrial enzyme in the presence of glycerol [10].

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Abbreviations: N_3-ATP, the 8-azido derivative of ATP; SL-ATP or SL-N_3-ATP, C3′-(2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid ester) of ATP and N_3-ATP, respectively; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography

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2. EXPERIMENTAL

N_3-ATP was synthesized according to Schäfer et al. [11], and was esterified with 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid to give SL-N_3-ATP essentially as described by Streckenbach et al. [3] for SL-ATP with the following modifications: after precipitation of the reaction product with acetone and centrifugation at 40000 × g, the mixture was triturated in the dark with methanol/ether, 40:60 (v/v). The yellowish, oily residue was dissolved in distilled water and lyophilized. Separation of the residual N_3-ATP was achieved by reversed-phase HPLC (LiChroprep RP 18, 6000 theoretical plates, 6 bar) with 0.1 M aqueous LiCl, containing 10% 2-propanol 3. SL-N_3-ATP elutes after about twice the volume required for the educt as monitored at 280 nm. It can be desalted by chromatography on Sephadex G10 with 0.1 M triethylammonium bicarbonate buffer, pH 7, as eluent. Fractions exhibiting both a 280 nm absorption and change in the refractive index were collected and lyophilized yielding SL-N_3-ATP in 35% overall yield. TLC: R_f = 0.4 on Nano-Sil-C_{18}-50 plates with 0.1 M LiCl, 10% 2-propanol 3 as eluent (N_3-ATP: R_f = 0.95) and R_f = 0 on Polygram CEL 300 PEI plates with 0.67 M LiCl as eluent (ATP: R_f = 0; ADP: R_f = 0.1; AMP: R_f = 0.4). IR: N_3 absorption at 2170 cm^{-1}. UV: λ_{max} = 282 nm, ϵ = 11650 cm^2/mmol.

For preparation of SL-ATP the original synthesis [3] was modified as described above for the azido derivative.

Nucleotide-depleted F_1-ATPase from beef heart mitochondria was isolated according to [12,13] and stored in 50% glycerol buffer (100 mM Tris-SO_4, 5 mM EDTA, pH 8.0) at −70°C. Protein concentration was determined according to [12,14], using BSA as a standard. ATPase activity was determined with the ATP regenerating system of Vogel and Steinhardt [15] or by measuring phosphate formation according to Arnold et al. [16].

Covalent labeling of F_1-ATPase with SL-N_3-ATP: 2.7 mg (7.5 nmol, based on M_r = 360000) of the enzyme stock solution was diluted with 0.5 ml of 10 mM Hepes buffer, pH 8, and reconcentrated to about 50 μl in a Centricon PM 30 concentrator at 5000 rpm for 30 min at 20°C, to remove any glycerol. Irradiation was carried out for 5 min in Duran vessels in a Ryonet photoreactor equipped with 16 concentric 350 nm lamps after addition of 41–495 nmol SL-N_3-ATP (5.5–66-fold molar excess) in 10 mM Hepes, pH 8, containing $MgCl_2$ in a concentration equimolar to the nucleotide.

Separation of the protein from free ligands was achieved according to Penefsky [17] by gel centrifugation chromatography on G50 fine for 2–5 min at 2000 rpm in a swing-out rotor. The ribose assay, as applied for determination of covalently bonded SL-N_3-ATP, was carried out according to Brückner [18] by the orcinol method with slight modifications as described by Wenzel [19].

ESR spectra were recorded with a Bruker ER-420 spectrometer, operating in the X-band mode at 15–20 mW microwave power and a peak to peak modulation amplitude of 0.8–2 G.

3. RESULTS AND DISCUSSION

Irradiation of SL-N_3-ATP leads to complete decomposition of the azido function upon irradiation at 350 nm within 5 min as monitored by the decrease of the 282 nm absorbance and shift to 275 nm. SL-N_3-ATP as well as SL-ATP itself exhibit a strongly immobilized ESR spectrum ($2A_{zz}$ = 66.5 G) in the presence of nucleotide-depleted F_1-ATPase [10] from beef heart mitochondria (fig.2A). The high field signal of the freely tumbling nucleotide is considerably broadened (fig.2A) as generally observed with spin-labeled compounds of this size [2].

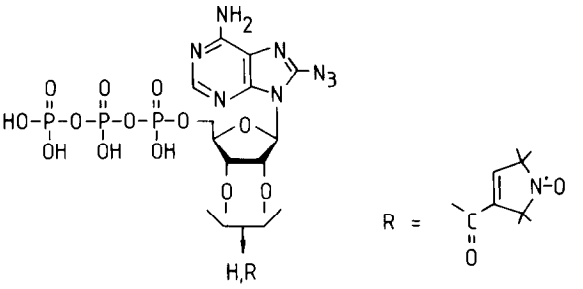

Fig.1. Structural formula of SL-N_3-ATP. The bracket indicates an equilibrium mixture of the 2′- and 3′-ester.

Irradiation of complexes of F_1-ATPase with SL-N_3-ATP led to a limiting covalent incorporation of

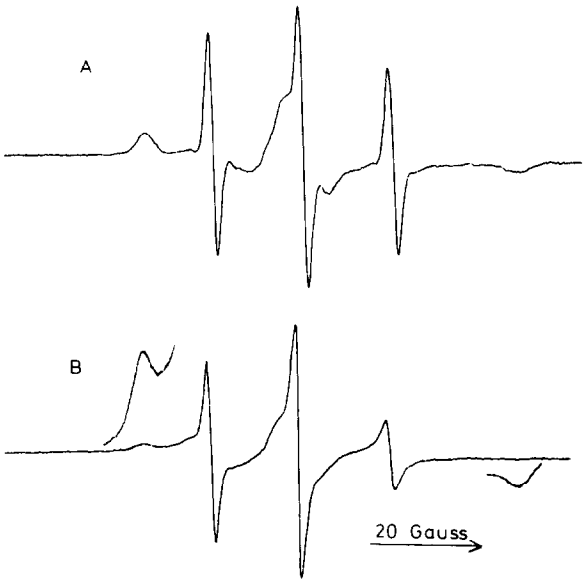

Fig.2. (A) ESR spectrum of 27 μM SL-N_3-ATP in a complex with 69 μM F_1-ATPase from beef heart in the presence of 37 μM $MgCl_2$ at 20°C. (B) After covalent incorporation of 3.5 equivalents into the enzyme. The high and low field signals in (B) are shown at 15-fold gain.

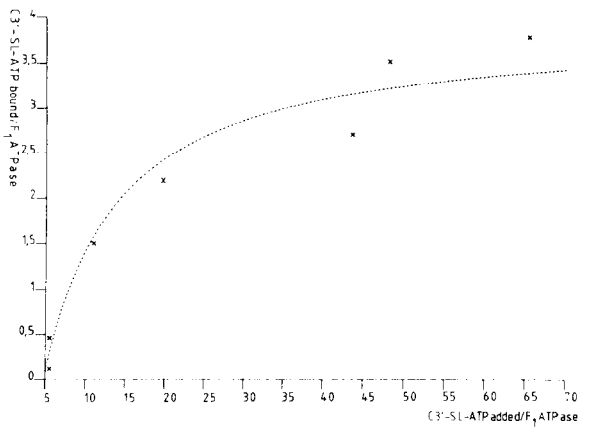

Fig.3. Covalent incorporation of SL-N_3-ATP into beef heart F_1-ATPase after irradiation for 5 min at 350 nm depending on the molar excess of the nucleotide. The enzyme exhibits 6 nucleotide-binding sites in its quaternary structure.

3.5 equivalents as revealed by ribose determination. A 60-fold excess of SL-N_3-ATP over the enzyme corresponding to a 10-fold excess per potential binding site was required (fig.3). The ESR spectrum again shows the spin-labeled nucleotide to be highly immobilized (fig.2B). A small fraction, however, appears to be bonded non-specifically to the surface of the enzyme as revealed by a 'free component' in the ESR spectrum (fig.2B). This mobile component could not be removed by repeated gel centrifugation chromatography. After correction for unspecific binding, about three equivalents of SL-N_3-ATP can be estimated to be covalently bonded to the nucleotide binding sites. This is supported by the fact that preincubation of the enzyme with either ATP and PP_i prevented incorporation of the immobilized species.

The covalently labeled enzyme retained about 50% of its activity, i.e., the hydrolysis of ATP, indicating that labeling has taken place primarily in the non-catalytic, tight sites [7–9].

Further addition of normal SL-ATP in large excess to covalently labeled F_1-ATPase gave rise to additional peaks in the high and low field region of the ESR spectrum (not shown). The outermost separation of the signals of 78 G at room temperature indicates dipolar interaction between two immobilized radicals separated by about 15 Å [5,20]. Hence, two nucleotide-binding sites appear to be in close vicinity. If indeed, covalent labeling had occurred in the tight sites, at least one loose site should be close. There is, however, an alternative possibility. An almost as large peak separation was attributed to hydrogen bonding of the nitroxide radical in the case of a spin-labeled peptide binding to leucine aminopeptidase [21].

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