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ATP binding to bovine serum albumin

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Specific binding of ATP to bovine serum albumin (BSA) is demonstrated employing ATP derivatives spin-labeled at either N^6 or C8 of adenine ring or at the ribose moiety. Based on a 1:1 stoichiometry binding constants are in the 50–100 μM range. Binding is largely competitive with ATP or stearic acid. A small fraction of the labeled nucleotides could not be liberated by these ligands. Binding of AMP is in the millimolar range, only.

Bovine serum albumin; ATP; Spin-labeled-ATP; Binding studies; Fatty acid; Doxyl-stearate

1. INTRODUCTION

Bovine serum albumin (BSA) is often added to dilute protein solutions in order to enhance their stability. BSA is thus considered rather inert in spite of a wide range of metabolites and other small molecules including many drugs binding to it [1,2]. In biophysical studies employing spin-labeled coenzymes and substrates of various biomolecules BSA has been used to mimic highly concentrated protein solutions and their effect on the ESR spectra of the free-tumbling fraction of the spin-labeled ligand [3]. In the course of corresponding experiments with various spin-labeled ATP derivatives we observed tight and specific binding of these compounds to BSA. Binding of ATP to BSA, to our knowledge, has not yet been described. BSA, however, has been utilized in several ATP-dependent systems as ionmotive ATPases, proteases and others, mainly to antagonize drug-induced effects (for examples, see [4–10]). Schraw and Post described covalent labeling of BSA by the dialdehyde derivative of ATP which was prevented by high concentrations of ATP. However, BSA served as a model compound for *non-specific* labeling as opposed to the specific interaction with Na^+,K^+-ATPase [11].

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Abbreviations: N^6-SL preceding AMP or ATP refers to their N^6-(2,2,6,6-tetramethyl-piperidin-4-yl-1-oxyl) derivatives; C8-SL preceding AMP or ATP refers to their 8-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl)amino derivatives; C2′,C3′-SL preceding ATP or AMP-PCP refers to the equilibrium mixture of their C2′ and C3′-(2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid esters); AMP-PCP is the β,γ-methylene triphosphate analog of ATP; BSA, bovine serum albumin; 5-Doxyl-stearic acid, 2-(3′-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinine-1-oxyl; HEPES, *N*-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

In this paper we describe the interaction of adenine nucleotide derivatives spin-labeled at N^6 or C8 of the adenine ring or the ribose moiety (N^6-SL-ATP, C8-SL-ATP, C2′,C3′-SL-ATP, Fig. 1) with BSA as studied by ESR spectroscopy.

2. MATERIALS AND METHODS

N^6-SL-ATP and C8-SL-ATP were synthesized according to [12] from the corresponding mononucleotides. N^6-SL-AMP was prepared according to [13] and C8-SL-AMP according to [14], with the modifications as described in [15]. C2′,C3′-SL-ATP was prepared from ATP according to [16] and as modified in [17] and C2′,C3′-SL-AMP-PCP as described in [18,19]. 5-Doxyl-stearic acid was purchased from Sigma Chemie GmbH (D-8024 Deisenhofen, Germany) and BSA from Boehringer Mannheim (D-6800 Mannheim, Germany).

ESR spectra were recorded with a Bruker ESP 300 spectrometer operating in the X-band mode. A microwave power of 6.3 mW and peak-to-peak modulation amplitudes of 0.8 Gauss were routinely employed. Measurements were performed at room temperature in micro flat cells [18] in total volumes of 50–60 μl of 50 mM HEPES buffer, pH 7.2. The fraction of bound spin-labeled nucleotides was calculated from the difference (decrease) in the amplitude of the high field line of spectra with and without protein [20]. Dissociation constants were evaluated according to Scatchard [21] and by non-linear regression analysis to theoretical binding curves by an iterative procedure [22]. For determination of binding constants C8-SL-ATP was varied from 50–670 μM at 44 μM BSA, N^6-SL-ATP from 40–200 μM at 100 μM BSA, and N^6-SL-AMP from 30–300 μM at 290 μM BSA.

3. RESULTS AND DISCUSSION

In the presence of BSA, the spin-labeled ATP analogs N^6-SL-ATP, C8-SL-ATP, C2′,C3′-SL-ATP and C2′,C3′-SL-AMP-PCP exhibit ESR spectra typical for highly immobilized nitroxide radicals with 2 A_{zz} values above 62 Gauss (N^6-SL-ATP: 62.0; C2′,3′-SL-ATP: 62.8 and C8-SL-ATP: 64.0; Fig. 2) Detailed binding studies yielded dissociation constants of 30 μM for C8-SL-ATP or 110 μM for N^6-SL-ATP and a stoichiometry of 1. Binding of C2′,C3′-SL-ATP and of C2′,C3′-SL-

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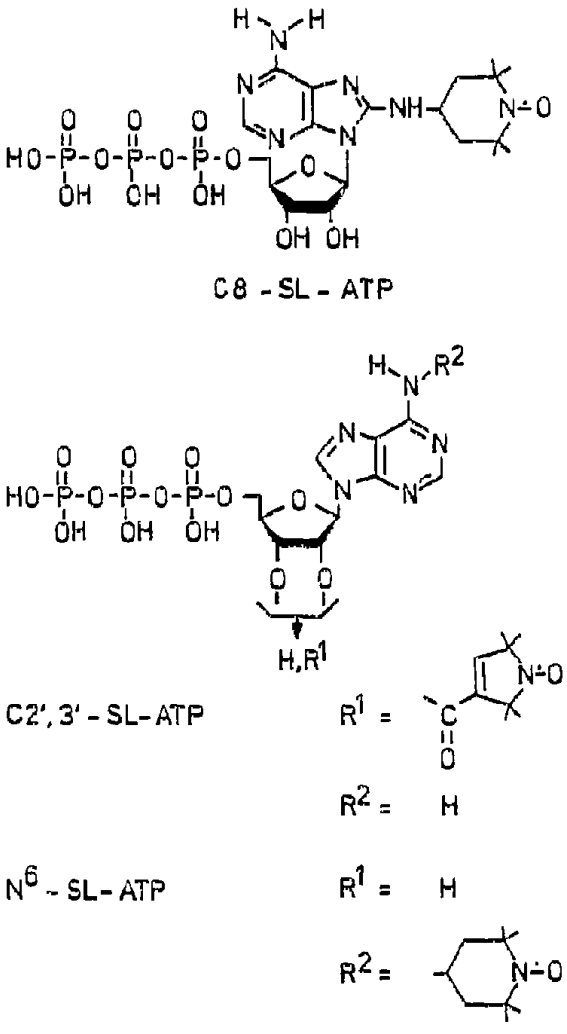

Fig. 1. Structural formulas of C8-SL-ATP, C2',C3'-SL-ATP and N^6-SL-ATP.

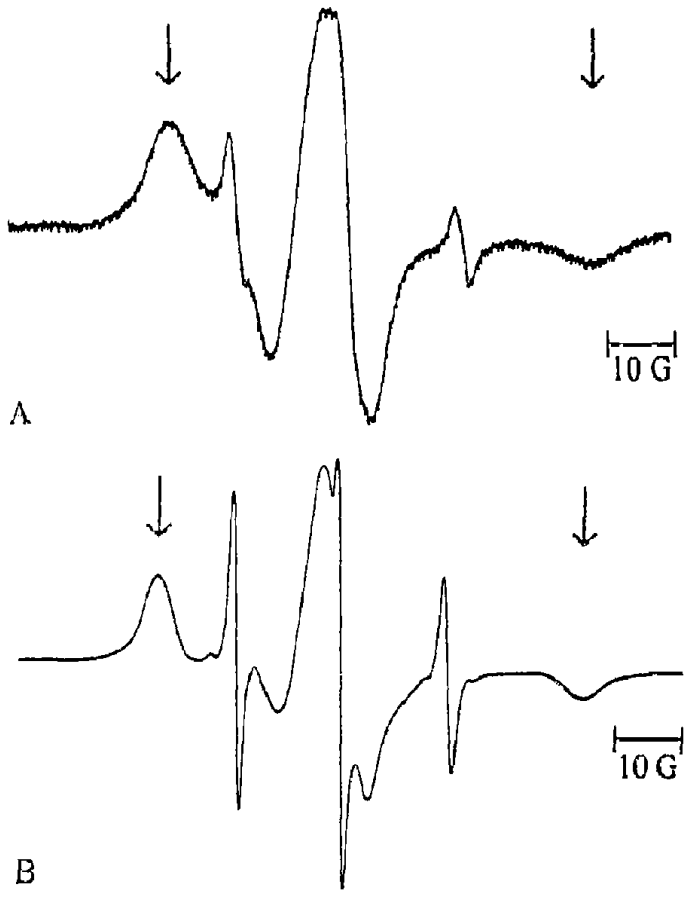

Fig. 2. ESR spectra of spin-labeled ATP derivatives in complex with BSA. 2 A_{zz} values are indicated by the arrows. (A) 170 μM C8-SL-ATP and 840 μM BSA in 80 mM HEPES buffer, pH 7.0 at 25°C, 2 A_{zz} = 64.0 Gauss. (B) 650 μM C2',C3'-SL-ATP and 880 μM BSA in 50 mM HEPES buffer, pH 7.2 at 20°C, 2 A_{zz} = 62.8 Gauss.

AMP-PCP, as estimated from data points obtained at selected concentrations, is comparable to that of C8-SL-ATP. ATP itself is able to replace most, but not all the bound spin-labeled ATP. At 100 μM BSA and N^6-SL-ATP each, 46% of the nucleotide were bound. Addition of equimolar amounts of ATP reduced this value to 27%, and a ninefold excess of ATP reduced it to 12%. However, further addition of ATP had almost no effect on the remaining fraction of bound N^6-SL-ATP.

Interaction of BSA with the monophosphates C8-SL-AMP or N^6-SL-AMP is considerably weaker with dissociation constants in the millimolar range, whereas binding of N^6-SL-ADP compares well with that of the corresponding triphosphate.

Since ATP-independent binding of N^6-SL-ATP could be due to the spin label itself, the interaction of 4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl (Tempamine) with BSA was examined. However, no reduction in the signal amplitude of the free-tumbling label could be detected in the presence of BSA within the concentration range studied (91–160 μM Tempamine at 91–830 μM BSA). A change of about 1% would have been detected.

A major physiological role of BSA is the transport of free fatty acids. Four mol of fatty acid are bound per mol of BSA [23]. These compounds are amphiphilic and hence, resemble nucleotides in this respect. Palmitate was chosen as an example to study their mutual interaction. Binding of N^6-SL-ATP (100 μM) to an equimolar amount of BSA alone was compared to BSA preincubated with a fourfold molar excess of palmitate. The latter showed a 50% reduction in the bound fraction, from 40 to 20 μM. Similarly, bound N^6-SL-ATP could be liberated by the addition of palmitate. At a ninefold molar excess, a residual 6%, only, of the spin-labeled nucleotide remained bound.

To further clarify a possible contribution of the spin label to binding affinity, ATP itself and a spin-labeled fatty acid, 5-Doxyl stearate, were employed in these competition experiments. BSA (30 μM) was incubated with an equimolar amount of 5-Doxyl stearate which led to an immobilization of 70% of the fatty acid. Addition of 100 μM ATP reduced this value to 40%. The

liberation of 9 μM 5-Doxyl stearate is quite significant when considering its four potential binding sites.

In conclusion, our data clearly show a specific interaction of ATP with BSA. Spin labels served as reporter groups of this interaction. Their location on the ATP molecule apparently is of minor importance and hence, the steric requirements for binding are rather low. This is somewhat surprising, because the labels become highly immobilized in complex with BSA in all cases. The hydrophobic spin label does indeed enhance the overall affinity of these analogs for the protein. In case of N^6-SL-ATP it could be shown that most of it binds competitively with respect to ATP itself. However, a minor fraction could not be liberated by either ATP or palmiate. Schön and Gnaiger from the Institute of Zoology at the University of Innsbruck have recently studied the interaction of ATP with BSA by differential scanning calorimetry and failed to detect significant energy changes (personal communication). However, in case of amphiphilic compounds showing both hydrophilic and strong hydrophobic interaction, the specific heat capacities can be temperature-independent.

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