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# Synthesis of spin-labeled 2-azido-ATP: evidence for distinct nucleotide-binding sites in calcium pump protein from sarcoplasmic reticulum

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A spin-labeled and photoreactive derivative of ATP was synthesized with the spin label attached to the 2'- or 3'-position of the ribose moiety and an azido group to C2 of the adenine ring (SL-2$N_3$-ATP). Irradiation of this compound at 350 nm generates a nitrene, which then reacts with nucleophiles in its vicinity. SL-2$N_3$-ATP, in the presence of $Ca^{2+}$, was hydrolyzed by the calcium pump protein ($Ca^{2+}$-ATPase) of fast twitch skeletal muscle sarcoplasmic reticulum. The SL-2$N_3$-ATP–enzyme complex in the absence of $Ca^{2+}$ exhibited strongly immobilized ESR spectra. ESR spectra obtained after covalent incorporation of SL-2$N_3$-ATP into $Ca^{2+}$-ATPase and removal of freely tumbling SL-2$N_3$-ATP exhibited motionally constrained species indicative of distinct and possibly adjacent ATP-binding sites. By contrast, with SL-ATP devoid of the azido group or with the corresponding 'non-cleavable' $\beta,\gamma$-methylene triphosphate analogue (SL-AMP-PCP), two distinct sites were not as well resolved in the ESR spectra due to spectral overlap with the signal from the freely tumbling fraction even with the enhanced spectral resolution provided by perdeuteration of the spin label. Thus, SL-2$N_3$-ATP may have general application for ESR studies of ATP-dependent proteins under conditions in which non-covalent interactions are too weak for motionally restricted species to be resolved.

Protein, $Ca^{2+}$ pump; ATPase, $Ca^{2+}$-; Photoaffinity labeling; Spin label; Perdeuteration; Azido-ATP, 2-

## 1. INTRODUCTION

8-Azido-ATP has found wide application as a photoaffinity reagent for various cation pumps (cation-motive ATPases). Due to the substituent at C8, the analog is preferentially in the *syn* conformation of the adenine ring with respect to the ribose moiety [1,2]. More recently, 2-azido-ATP has become the preferred reagent, since ATP was found to combine with several proteins in the *anti* conformation [3–5].

Recently, we introduced photoaffinity spin-labeled derivatives of $NAD^+$ [6] and ATP [7]. Such analogs are useful probes for ESR investigations under conditions in which non-covalent binding is too weak for the bound nucleotide to be detected by ESR spectroscopy, i.e. the ESR spectra are dominated by the high-amplitude signal of the free component. In addition to SL-8$N_3$-ATP [7], we have now synthesized the corresponding SL-2$N_3$-ATP (fig.1). With these photoaffinity reagents, the ESR spectral characteristics of bound ligand can be determined after covalent incorporation followed by separation from the free ligand.

The calcium pump protein ($Ca^{2+}$-ATPase) of the sarcoplasmic reticulum membrane consists of a single type polypeptide chain of $M_r$ 110331 (1001 amino acids) [8]. The enzyme catalyzes the active

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*Abbreviations:* 2$N_3$ (8$N_3$), 2-azido (8-azido) derivative of ATP; SL-, spin label [C3'-(2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid ester)] derivatives, in which the SL is at the 2'- or 3'-hydroxyl of the ribose; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; Mes, 4-morpholineethanesulfonic acid; SR, sarcoplasmic reticulum



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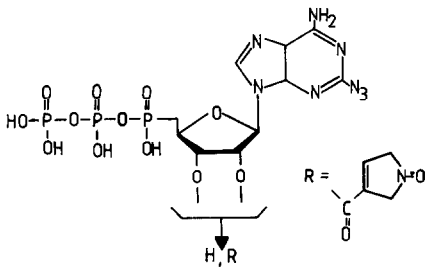

Fig.1. Structural formula of SL-2$N_3$-ATP. The bracket indicates an equilibrium mixture of 2'- and 3'-esters.

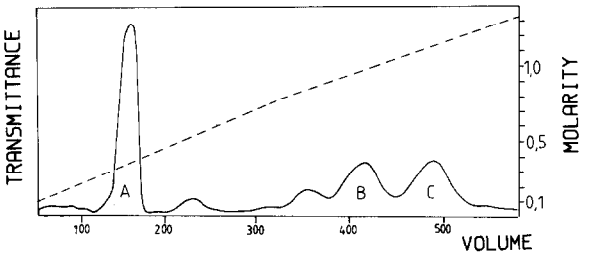


Fig.2. Typical elution profile for the purification of SL-2$N_3$-ATP on DEAE-Sephadex A25. A linear gradient of 0–1.3 M triethylammonium bicarbonate (600 ml) was used. Peaks were assigned by TLC and ESR analysis; see text. (A,C) Unreacted starting materials, SL and 2-azido-ATP, respectively; (B) product, SL-2$N_3$-ATP. The elution profile for the purification of SL-2$N_3$-ATP is very similar. The transmittance is at 264 nm.

transport of $Ca^{2+}$ coupled to the hydrolysis of ATP with a $Ca^{2+}$/ATP stoichiometry of two [9–11]. There are two ATP-binding sites. The high-affinity site ($K_m$ in the micromolar range) is associated with catalysis. The low-affinity site ($K_m$ in the submillimolar [12] or millimolar range [13]) appears to have a modulatory role [14–16]. ESR binding studies with SL-ATP exhibited only a single type of immobilized component [13]. The interpretation of these spectra, however, was complicated by a large ESR spectral component arising from the unbound and freely tumbling SL-ATP and SL-ADP (formed by enzymic hydrolysis) present in the samples. Here, we have covalently bound SL-2$N_3$-ATP to the calcium pump protein of the SR and are now able to resolve two spectral components for the bound ligand.

## 2. MATERIALS AND METHODS

2-Azido-ATP was synthesized from 2-chloroadenosine essentially according to Czarnecki [17]. Esterification of 32 mg (40 $\mu$mol) 2-azido-ATP (triethylammonium salt) with 14 mg (76 $\mu$mol) 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid was carried out as previously described for SL-8$N_3$-ATP [7] to give SL-2$N_3$-ATP in 18% yield. However, purification was significantly simplified by anion-exchange chromatography (fig.2) on DEAE-Sephadex A25 using triethylammonium bicarbonate buffer (pH 7.3) as eluent. Removal of excess salts was achieved by repeated co-evaporation with methanol and subsequent lyophilization. The purified SL-2$N_3$-ATP had the following properties: the ESR spectrum showed an amplitude ratio (high-field to center-field peak) of 0.6. (UV) $\epsilon$ = 15 500 $cm^2$/mmol in methanol, $\epsilon$ = 10 300 $cm^2$/mmol in an aqueous solution at pH 7.0, and $\lambda_{max}$ = 270 nm. (IR) $N_3$ absorption at 2130 $cm^{-1}$; (TLC) $R_f$ = 0.56 on silica gel 60 $F_{254}$ plates (Merck, Darmstadt) using isobutyric acid/conc. ammonia/water (66:1:33, v/v) as solvent system and $R_f$ = 0.39 (0.79 for unlabeled 2$N_3$-ATP) with 10% isopropanol, 90% 0.1 M LiCl as eluent on $RP_{18}$-$F_{254}$ plates (Merck). (HPLC) Ultropac column Lichrosorb RP 18.5 $\mu$m (4 × 250 mm) (LKB, Bromma), eluent 10% isopropanol, 90% 0.1 M LiCl (pH 7.0). SL-2$N_3$-ATP was stable for at least 3 months upon storage at −70°C in aqueous solution as monitored by HPLC.

SL-ATP was prepared as in [7,18]. The corresponding analog with a perdeuterated spin label was obtained by the same procedure with 2,2,5,5-[$^2H_{13}$]tetramethylpyrroline-1-oxyl-3-carboxylic acid (MSD Isotopes, Montreal) as starting material. Perdeuterated SL-AMP-PCP was prepared from the same spin label and AMP-PCP following a general procedure by Jeng and Guillory [19].

ESR spectra were recorded on a Bruker ESP 300 spectrometer (X-band) with a modulation frequency of 100 kHz, a modulation amplitude of 1.0 G, a microwave power of 6.3 or 12.6 mW and a sweep width of 100 or 120 G. The measurements were performed at 25°C in a total volume of approx. 60 $\mu$l of 80 mM Hepes (pH 7.4), 80 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 80 $\mu$M EGTA (buffer I); 100 mM Mes (pH 7.2), 5 mM $MgCl_2$, 10 $\mu$M $CaCl_2$, 20 $\mu$M EGTA and 150 mM sucrose (buffer II) or 80 mM Hepes (pH 6.8), 80 mM KCl, 100 $\mu$M EGTA, 100 $\mu$M $MgCl_2$ (buffer III). Further details are given in table 1.

Longitudinal tubules of SR (fraction $I_{32}$ from the 32.5–34.5% sucrose layer) were prepared from rabbit fast twitch skeletal muscle by zonal centrifugation on a sucrose density gradient [20]. It is composed of one membrane type, the calcium pump membrane. About 90% of the protein of this highly specialized membrane is the calcium pump [20]. The calcium pump protein concentration was calculated using a value of 8.1 nmol per mg protein. Protein concentration was measured according to Lowry et al. [21], using bovine serum albumin as standard. ATPase activity was assayed spectrophotometrically at 340 nm following the oxidation of NADH in a coupled system with pyruvate kinase and lactate dehydrogenase [22] or by monitoring the change in pH value upon ATP hydrolysis [23].

Photolabeling procedure: A suspension of 8.2 mg/ml of longitudinal tubules of SR and 1.68 mM SL-2$N_3$-ATP in a total volume of 70 $\mu$l of 0.3 M sucrose, 10 mM $MgCl_2$, 1 mM EGTA, 80 mM KCl, pH 7.0, was irradiated 3 times for 5 min each in a Ryonet photoreactor (Middletown, CT) equipped with 16 con-

Table 1

Binding of various spin-labeled ATP derivatives to calcium pump protein from SR

| Fig. | [Enzyme] ($\mu$M) | [$Ca^{2+}$] (mM) | [Spin-labeled nucleotide] ($\mu$M) | [Enzyme-nucleotide complex] ($\mu$M) | $K_d$[a] (mM) |
|---|---|---|---|---|---|
| 3A | 370 | 1 | 3200[c] | 240 | 1.60 |
| 3B | 770 | - | 630[d] | 155 | 1.88 |
| [b] | 760 | 10 | 620[d] | 220 | 0.98 |
| 3C | 660 | 10 | 60[d] | 25 | 0.89 |
| 4A | 250 | 0.01 | 88[e] | 30 | 0.43 |
| 4B | 180 | 0.01 | 990[e] | 140 | 0.32 |
| 4C | 75 | 0.01 | 1680[e] | 37[f] | - |

[a] Experiments were not designed to quantitate $K_d$. Values are approximate only
[b] ESR spectrum not shown
[c] SL-ATP
[d] SL-AMP-PCP
[e] SL-$2N_3$-ATP
[f] After irradiation and gel centrifugation

centric 350 nm lamps. 30-min intervals were left between the irradiation periods to allow for re-equilibration of the azido and tetrazolo forms of SL-$2N_3$-ATP [17,24]. Separation of the protein from unbound spin-labeled nucleotide was achieved according to Penefsky [25] by repeated gel-centrifugation chromatography on Sephadex G-50 fine (Pharmacia, LKB, Freiburg) for 5 min at 2000 rpm (600 × g) in a swing-out rotor (Hettich Universal, Tuttlingen).

## 3. RESULTS AND DISCUSSION

ATP binding by the calcium pump protein from SR was investigated by using derivatives of ATP, AMP-PCP and $2N_3$-ATP spin-labeled at the ribose moiety. SL-ATP is hydrolyzed by the enzyme at about 50% the rate of ATP, per se, independent of whether determined in a coupled assay with pyruvate kinase and lactate dehydrogenase or simply by following the pH change upon ATP hydrolysis. Oliveira et al. [13] had previously reported SL-ATP not to be a substrate in a coupled assay system, in which the concentrations of individual components were different. In particular, the concentration of pyruvate kinase, which has to phosphorylate SL-ADP, is 10-fold higher in our coupled assay system. In that work [13] as well as in studies in our laboratory [26], the ESR spectra of SL-ATP with the transporter were found to be dominated by the freely tumbling component referable to unbound nucleotide.

In order to provide improved ESR spectral resolution so as to look for potentially distinct ATP-binding sites, SL-ATP was synthesized with a perdeuterated spin label. The advantage of the enhanced spectral resolution provided by such analogs is well established [27,28]. At the high excess of analogs required for combining with the low-affinity regulatory site, the amplitude of the free signal was still too large for detailed analysis of the motionally restricted species in the spectra (fig.3A). We obtained some improvement in resolution by utilizing the non-hydrolyzable SL-AMP-PCP (fig.3B vs A). In this way, formation of SL-ADP, which binds only weakly, is precluded (see also [13]). Spectra recorded in either the presence or absence of $Ca^{2+}$ at various transporter/nucleotide ratios were similar in line shape but binding was slightly tighter in the presence of up to 10 mM $Ca^{2+}$ (for details see table 1). It should be noted that the low-field signal region of the ESR spectral component referable to bound SL-AMP-PCP is asymmetric (arrow 2, fig.3C), suggesting the presence of more than one bound component. This asymmetry appears to be more pronounced in the presence of $Ca^{2+}$ even at a 10-fold excess of the calcium pump protein (fig.3C). Moreover, this spectrum also shows a corresponding peak in the high-field region separated by 76 G (arrows 2′ and 2).

SL-$2N_3$-ATP is hydrolyzed by the $Ca^{2+}$-ATPase at 13% of the rate of ATP. Fig.4A shows an ESR spectrum of a complex of this ligand with the enzyme in which 32% of the SL-$2N_3$-ATP contributes to the motionally restricted enzyme-bound species ($2A_{zz}$ = 66.5 G, distance between arrows 1 and 1′). Addition of a 10-fold excess of ATP resulted in loss of the bound signal and a corresponding increase in the free component, i.e. SL-$2N_3$-ATP is bound to the ATP site(s) exhibiting an apparent $K_d$ of about 0.6 mM.

At a 5-fold molar excess of SL-$2N_3$-ATP to enzyme, the additional component in the low-field region, as detected with SL-AMP-PCP (fig.3B,C) is resolved (fig.4B). This spectral component was also detected in the absence of $Ca^{2+}$ (not shown) and, as with SL-AMP-PCP, is therefore independent of the presence of $Ca^{2+}$. The tight binding of ATP to the calcium pump protein does not require $Ca^{2+}$, which is essential for the hydrolysis of ATP [29].

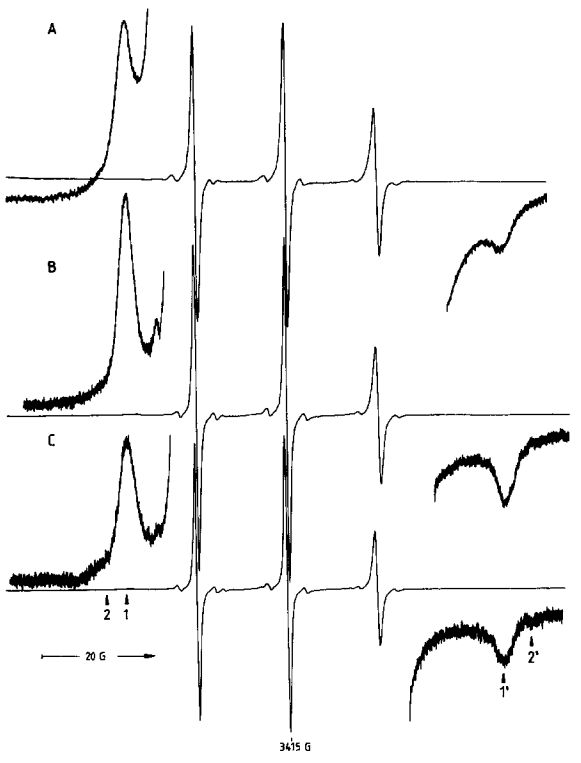


Fig.3. ESR spectra of SL-ATP (A; buffer I) and SL-AMP-PCP (B,C, buffer III; C, addition of $Ca^{2+}$) in complexes with calcium pump protein from SR. Concentrations given in table 1. Spectra were recorded with the following maximal relative gain settings: A/B/C = 2:1:5.

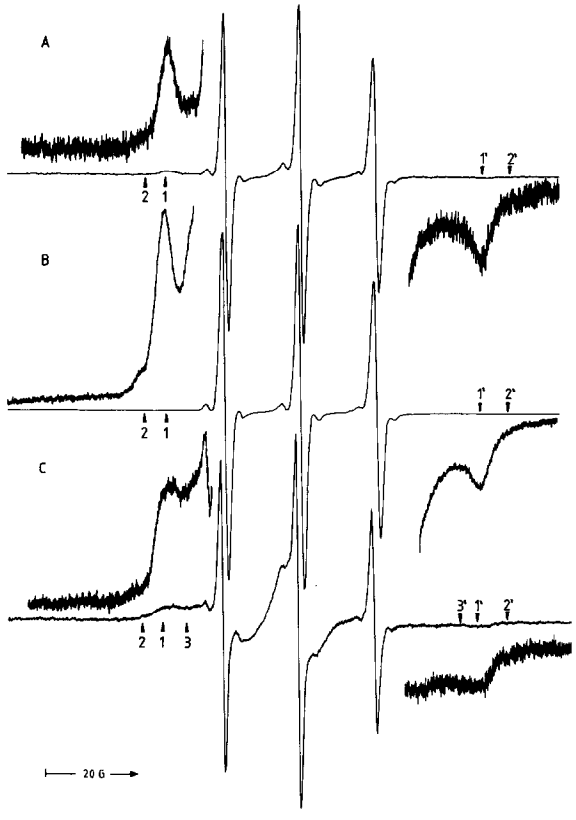


Fig.4. ESR spectra of SL-2$N_3$-ATP nucleotide with calcium pump protein from SR (A,B, buffer I; C, buffer II). Concentrations given in table 1; maximal gain settings: A/B/C = 3:1.25:3.75. Arrows indicate distinct motionally constrained components. Separation between band 1 (arrows 1,1'), 66.5 G; band 2 (arrows 2,2'), 76–77 G; band 3 (arrows 3,3'), 60–61 G.

The ESR spectrum after covalent incorporation of SL-2$N_3$-ATP into the calcium pump protein is shown in fig.4C. Labeling was carried out using a 20-fold molar excess of the nucleotide and the non-covalently bonded SL-2$N_3$-ATP was removed by gel centrifugation [25]. Irradiation of the transporter alone under identical conditions did not lead to a significant loss in activity (<4%). About 0.5 + 0.1 equiv. were bound per mol calcium pump protein as judged by ESR spectroscopy. In the absence of the intense and overlapping spectral component referable to free ligand, the spectrum reveals additional bands in the low- and high-field regions separated by 60–61 G (fig.4C, arrows 3 and 3'). Some residual free signal present in the sample may be due to covalent binding at non-specific sites at the surface of the enzyme. The complex line shape could arise from distinct and structurally different binding sites on the calcium pump which comprises 90% of the total protein present. The spectral components are too large to arise from binding to a minor contaminant in the residual protein. The observed splitting of 60–61 G, however, is rather small for a separate bound component but could arise from spin-spin interaction between SL-2$N_3$-ATP molecules bound to adjacent sites on the enzyme. A separation of nitroxide spins of 12–15 Å would yield this type of spectrum, in which both the original low- and high-field signals are split into nearby bands, separated by about 8.5 G (between arrows 2 and 3, fig.4C). Corresponding splittings of the central line are still obscured by the free component. Such dipolar interactions have been observed previously for SL-NAD bound to adjacent subunits in glycer-

aldehyde-3-phosphate dehydrogenase and were analyzed in terms of a structural model [27,30]. Corresponding studies are now being carried out with SL-2$N_3$-ATP covalently bound to the calcium pump protein in order to determine the origin of the complex ESR spectral line shape. SL-2$N_3$-ATP, the synthesis of which is described here, may have general application in ESR studies of ATP-dependent proteins under conditions in which non-covalent interactions are too weak for motionally restricted species to be readily characterized. With $F_1$-ATPase from thermophilic bacteria, covalent labeling of four out of six nucleotide-binding sites has been achieved (Trommer et al., in preparation).

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