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Research Letter

Saturation transfer EPR measurements of the rotational motion of a strongly immobilized ouabain spin label on renal Na,K-ATPase*

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The rotational motion of an ouabain spin label with sheep kidney Na,K-ATPase has been measured by electron paramagnetic resonance (EPR) and saturation transfer EPR (ST-EPR) measurements. Spin-labelled ouabain binds with high affinity to the Na,K-ATPase with concurrent inhibition of ATPase activity. Enzyme preparations retain 0.61 ± 0.1 mol of bound ouabain spin label per ATPase $\alpha\beta$ dimer. The conventional EPR spectrum of the ouabain spin label bound to the ATPase consists almost entirely (>99%) of a broad resonance which is characteristic of a strongly immobilized spin label. ST-EPR measurements of the spin labelled ATPase preparations yield effective correlation times for the bound labels of 209 ± 11 μs at 0°C and 44 ± 4 μs at 20°C. These rotational correlation times most likely represent the motion of the protein itself rather than the independent motion of mobile spin probes relative to a slower moving protein. Additional ST-EPR measurements with glutaraldehyde-crosslinked preparations indicated that the observed rotational correlation times predominantly represented the motion of entire Na,K-ATPase-containing membrane fragments, rather than the motion of individual monomeric or dimeric polypeptides within the membrane fragment. The strong immobilization of the ouabain spin label will make it an effective paramagnetic probe of the extracellular surface of the Na,K-ATPase for a variety of NMR and EPR investigations.

Electron paramagnetic resonance (EPR); Saturation transfer EPR; Na,K-ATPase; Ouabain; Ion transport; ATPase rotational motion

1. INTRODUCTION

Na,K-ATPase (EC 3.6.1.3, ATP phosphohydrolase), is a plasma membrane transport system which is essential for normal cell function in all mammalian cells. Much effort has been devoted to understanding how the structure and dynamics of the Na,K-ATPase affect the mechanism of energy coupling and Na^+ and K^+ ion transport. For example, Esmann et al. [1] have recently studied the rotational motion of a maleimide spin-labelled Na,K-ATPase in its membrane environment using conventional EPR and saturation transfer EPR in an attempt to characterize the molecular organization of the Na,K-ATPase in the membrane and correlate enzyme activity data with rotational correlation data. However, the heterogeneous nature of the maleimide spin label bound to the Na,K-ATPase made the interpretation of the EPR data ambiguous. Tightly bound labels reported only the microsecond motion of the protein itself, while another population of bound labels were less immobilized and reported their own nanosecond motion independent of the protein. Since even small fractions of nanosecond motion quickly dominate such two component spectra, they must be interpreted with great care [2].

The obvious solution to this problem would be to develop a spin label which binds tightly to the Na,K-ATPase at a single site and is totally immobilized by the protein. Such a label would provide singular information about the rotational dynamics of the protein system and the structure of the protein in the vicinity of the label binding site. An additional and longstanding goal in our laboratory has been to develop a paramagnetic probe of the extracellular surface of the Na,K-ATPase for EPR and NMR studies of structure and function.

The Na,K-ATPase is acknowledged to be the primary pharmacologic receptor for cardiac glycosides [3,4]. The affinity of the cardiac glycoside receptor and the kinetics of glycoside binding have been studied in detail [5-7]. One cardiac glycoside binds per $\alpha\beta$ subunit of the Na,K-ATPase with a nanomolar dissociation constant

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Abbreviations: Na,K-ATPase, sodium and potassium ion-activated adenosinetriphosphatase; Tris, tris(hydroxymethyl)aminomethane; 4-amino-Tempo, 4-amino-2,2,6,6-tetramethylpiperidino-oxyl; OSL, ouabain spin label; MSL, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; ATP, adenosine 5'-triphosphate

* The Na,K-ATPase preparations used here consisted of membrane fragments containing the ATPase

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[8]. Solomonson and Barber [9] have described the synthesis of a novel nitroxide derivative of the cardiac glycoside ouabain. We have initiated a series of conventional and ST-EPR studies of the interaction of this ouabain derivative with the Na,K-ATPase.

Conventional EPR spectra are sensitive to molecular motions on the nanosecond timescale, but ST-EPR spectra are sensitive to motions in the microsecond to millisecond timescale [2,10]. Our results indicate that the OSL binds in a manner similar to ouabain, and that the nitroxide moiety is completely immobilized in this complex, making this spin label suitable for use in EPR and ST-EPR studies of the microsecond timescale rotational motions of the Na,K-ATPase.

2. MATERIALS AND METHODS

2.1. *Materials*

Ouabain octahydrate, sodium cyanoborohydride, sodium periodate, sodium phosphate, Trizma hydrochloride, imidazole, ATP, and hemoglobin used were purchased from Sigma Chemical Company. 4-amino-Tempo was purchased from Aldrich Chemical Company. The spin-labelled ouabain was synthesized according to Solomonson and Barber [9]. Buffers and appropriate reagent solutions were further purified by column treatment with Chelex-100 (Bio-Rad) to remove metal contaminants.

2.2. *Enzyme preparation and assay*

Na,K-ATPase was purified as membrane fragments from microsomal fractions of fresh sheep kidney outer medulla as described by Jørgensen [11] with modifications as described by O'Connor and Grisham [12].

2.3. *Spin labelling of hemoglobin with maleimide spin label*

The procedure for spin labelling hemoglobin was adapted from Hemminga et al. [13]. The modifications to the original procedure have been described elsewhere [14].

2.4. *Spin labelling of Na,K-ATPase with spin-labelling ouabain*

The Na,K-ATPase (2.5–3 mg/ml) was incubated with 1 mM spin-labelled ouabain in 10 mM Tris, pH 7.2, containing 3 mM ATP, 3 mM $MgCl_2$, 120 mM NaCl for 1 h at room temperature. Unbound spin label was separated from the spin-labelled Na,K-ATPase by washing the membranes three times with 30 ml of 150 mM NaCl, 15 mM imidazole, pH 7.2, centrifuging each time at $25000 \times g$ for 35 min. Following the final wash to remove unbound label, the pellet was resuspended in 150 μl of 150 mM NaCl, 15 mM imidazole, pH 7.2, and pelleted in a Beckman Airfuge at maximum speed for 10 min. Following the removal of excess buffer, the pellet, which was approximately 75 mg/ml, was drawn by gentle suction into a special, gas permeable capillary sample cell made of TPX as described by Popp and Hyde [15]. Enough sample was drawn into the TPX such that the sample length was 20 mm approximating a line sample [2,16]. Typical sample volumes were ~20 μl. The TPX sample tube and holder were held in place in the variable temperature dewar by means of a specially designed delrin shim scribed with a notch sufficient to allow the cooled nitrogen gas was allowed to flow out of the assembly. The TPX assembly was placed into the cavity and cooled nitrogen gas was allowed to flow over the sample for at least 60 min to allow removal of dissolved molecular oxygen [15]. Neither the shim nor TPX holder extended into the cavity, which thus contained only the sample in the TPX tubing.

2.5. *Electron paramagnetic resonance measurements*

All EPR experiments were performed on a Varian E-109 Century Series X-band spectrometer using an E-231 cavity (TE_{102} mode) with temperature control provided by a Varian E321 cavity dewar insert equipped with a heater-sensor probe. Rotational tumbling times (τ_r) were estimated from conventional EPR spectra using the relation:

$$\tau_r = 6.5 \times 10^{-10} W_0 ((h_0/h_{-1})^{1/2} - 1 \quad (1)$$

where W_0 is the peak-to-peak line width of the central nitroxide peak, in Gauss, and h_0 and h_{-1} are the peak-to-peak line heights of the central and low field peaks, respectively. The method for determining τ_r values from ST-EPR spectra was taken from Squier and Thomas [2]. In the present paper, the normalized intensity parameters, $\int V_1^2$ and S_i are used. ST-EPR standard curves for both methods were constructed using reference spectra obtained from maleimide spin-labelled hemoglobin in various glycerol-water mixtures as described earlier. Values for τ_r calculated for the reference samples were obtained using a radius of 29 Å for hemoglobin [17] and the viscosity values for the solutions were taken from Segur and Oberstar [18], and Slie et al. [19].

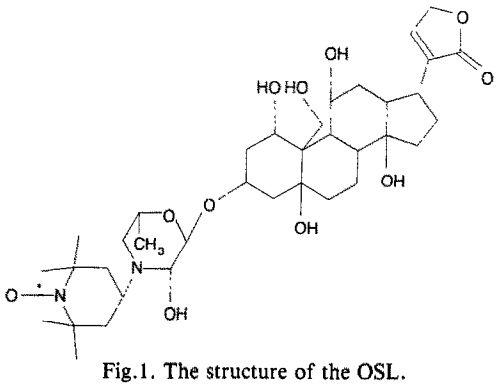

Fig.1. The structure of the OSL.

3. RESULTS AND DISCUSSION

3.1. *Interaction of spin label ouabain with Na,K-ATPase*

The structure of the OSL used in these experiments is shown in fig.1. The spin label was synthesized essentially according to Solomonson and Barber [9], and the

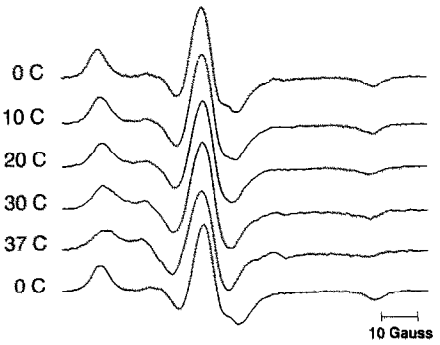

Fig.2. Temperature dependence of V_1 EPR spectra of spin-labelled ouabain to the Na,K-ATPase. Spectra are characterized by a decreasing maximum hyperfine splitting, $2T_{\parallel}'$, an increasing half-width at half-width of the low field resonance, Δh_L, and an increasing mole fraction of weakly bound spin labels with increasing temperature. 0°C rpt signifies cooling the sample back to 0°C after recording the 37°C spectrum. Spectra were recorded with microwave field intensity, H_1, of 0.032 Gauss, 100 Gauss scan range, 100 kHz modulation and detection, and 2 Gauss modulation amplitude at a minimum rate of 25 Gauss/min and a maximum time constant of 0.5 s. Spectra were normalized to the same spin concentration and represent 100 Gauss sweep widths.

structure was confirmed by mass spectrometric analysis. Since ouabain binds to Na,K-ATPase in the E_2-P conformation [20–22], the ATPase was incubated with the spin label in the presence of 3 mM ATP, 3 mM $MgCl_2$, and 120 mM NaCl. As previously reported [9], the OSL inhibited the Na,K-ATPase with approximately the same efficacy as ouabain itself, and the binding of the spin label was accompanied by a stoichiometric loss of enzymatic activity. From a comparison of the integrated intensities of the spin-labelled ATPase and standard spectra, a binding stoichiometry of 0.61 ± 0.1 mol of spin label bound per $\alpha\beta$ diprotomer was calculated, consistent with results obtained in binding studies with normal ouabain.

3.2. *Conventional EPR analysis of OSL-Na,K-ATPase*

The spectrum of the OSL bound to the Na,K-ATPase is initially composed of a broad resonance, typical of a highly immobilized spin label ($\tau_r \geq 10^{-6}$ s), which accounts for 97% of the total signal, and a narrow resonance, typical of weakly immobilized nitroxides ($\tau_r \sim 10^{-9}$ s), which accounts for 3% of the total signal. The mole fraction of the weakly immobilized component decreased with time to a level of 0.5 ± 0.1% of the total signal. A spectrum of the spin-labelled ouabain bound to the Na,K-ATPase is shown in fig. 2 (top spectrum). The loss of the weakly immobilized component was accompanied by an overall loss of spectrum intensity of 2.9 ± 0.2%, and spectral intensity and lineshape

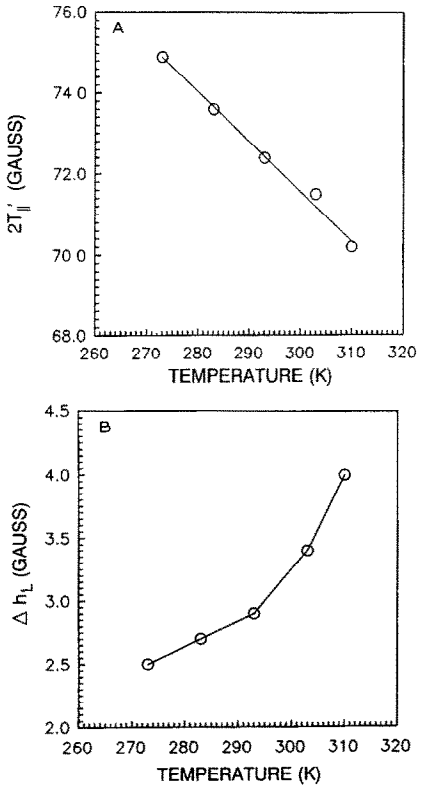

Fig.3. Temperature dependence of conventional EPR spectral parameters of the OSL-Na,K-ATPase: (A) $2T_{\parallel}'$ and (B) Δh_L.

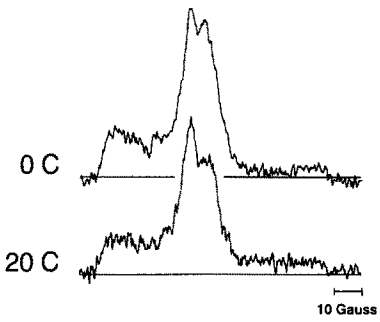

Fig.4. ST-EPR V_2' spectra of the OSL-Na,K-ATPase at 0°C and 20°C. Spectra were collected using H_1 = 0.25 and were normalized to the same spin concentration and gain. Other conditions included 100 Gauss scan range, 50 kHz modulation and 100 kHz detection set 90° out-of-phase, and 5 Gauss modulation amplitude at a minimum rate of 6.25 Gauss/min and maximum time constant of 2 s. Proper adjustment of the out-of-phase detection condition was done by the self-null method, and proper selection and determination of H_1, the intensity of the microwave field at the sample, was done by comparison with peroxylamine disulfonate, both as described by Squier and Thomas [2]. Baselines represent 100 Gauss.

remained constant beyond this point. The loss of the weakly immobilized component may be the result of preferential reduction of the weakly bound labels or of non-specific hysteresis of all labels as the weakly immobilized labels bind more tightly.

3.3. *Temperature dependence of spin-labelled Na,K-ATPase spectra*

The spectrum of the ATPase bound OSL was temperature dependent, as shown in fig.2. At 0°C, the strongly immobilized component of the spectrum was dominant (~100%), the splitting between the low and high field extrema ($2T_{\parallel}'$) was at a maximum (74.9 Gauss), the half-width at half height of the low field peak (Δh_L) was at a minimum (2.5 Gauss) and the mole percent of the weakly immobilized component was at a minimum (~0.8%). As the temperature was increased, $2T_{\parallel}'$ decreased and Δh_L increased, as shown in fig.3. At every temperature measured, however, the spectrum remained characteristic of a strongly immobilized label. When the sample temperature was reduced to 0°C, $2T_{\parallel}'$ and Δh_L returned to their original values, but the mole fraction of the weakly immobilized component had increased to 1.5% (data not shown). This irreversible temperature behavior of the weakly immobilized probes may be due to the loosening of bound labels due

Table 1

Normalized intensity parameters and corresponding τ_r values for saturation transfer spectra of spin-labelled ouabain bound to the Na,K-ATPase

Temperature (°K)	$\int V_2'$	S_i'
273	0.73 ± 0.02	0.81 ± 0.02
(τ_r)	(208 ± 10 μs)	(208 ± 11 μs)
293	0.56 ± 0.01	0.56 ± 0.01
(τ_r)	(45 ± 5 μs)	(45 ± 5 μs)

to an initial temperature-induced denaturation of a small percentage of OSL binding sites.

3.4. *Saturation transfer analysis of spin-labelled Na,K-ATPase*

Since the conventional spectra of the OSL bound to the Na,K-ATPase were indicative of a label in a single, highly immobilized site, and almost devoid of contributions from weakly immobilized probes (below 20°C), the OSL bound to the ATPase was considered to be an adequate probe for studying the rotational motion of the protein by ST-EPR.

The ST-EPR V_2' spectra of the OSL bound to the Na,K-ATPase at 0°C and 20°C are shown in fig.4. The spectra contained marked changes in lineshape detail in the 20°C spectrum as compared to the 0°C spectrum, typical of increased rotational mobility of the bound label at the higher temperature. The spectra were characteristic of a highly immobilized spin label and contained less than 1% contribution from weakly immobilized probes (based on conventional spectral analysis).

ST-EPR V_2' spectra, being second harmonic, out-of-phase, are generally ten times less intense than corresponding first harmonic, in-phase V_1 spectra. This, in combination with the low concentration of spin label in a given sample (generally 75–125 μM), the signal-to-noise ratios of V_2' spectra of OSL bound to the Na,K-ATPase were extremely low. Spectral analysis by the lineheight method was considered ineffective since the standard deviations of such ratios brought errors of >50% in τ_r estimation. There was, however, appreciable V_2' intensity allowing spectral analysis and τ_r estimation by the V_2' integrated intensity method [4]. At temperatures above 0°C, the signal-to-noise of the V_2' spectra decreased introducing errors into the integrations of the V_2' spectra. To serve as a complementary measure of OSL-Na,K-ATPase rotational correlation times, ST-EPR spectra were also analyzed by the V_1 intensity parameter, denoted S_i' [2]. Conventional V_1 spectra were collected for each sample at low, non-saturating power (H_1 = 0.032 Gauss) and at high, saturating power (H_1 = 0.25 Gauss). The spectra were normalized as described in section 2 to produce the S_i' parameter. Rotational correlation times were estimated from the S_i' vs τ_r standard curve constructed from aqueous glycerol MSL-hemoglobin samples (not shown). The result of these measurements is shown in table 1.

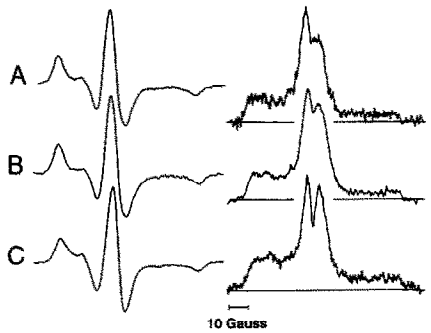

Fig.5. Conventional and ST-EPR spectra of control and glutaraldehyde-crosslinked OSL-Na,K-ATPase at 20°C. (A) Control OSL-Na,K-ATPase (τ_r = 45 ± 5 μs). (B) OSL-Na,K-ATPase treated with 1% glutaraldehyde for 10 min (τ_r = 220 ± 10 μs). (C) OSL-Na,K-ATPase treated with 1% glutaraldehyde for 12 h (τ_r = 100 ± 5 μs). Conventional EPR spectra were collected with H_1 = 0.032 Gauss and ST-EPR spectra were collected with H_1 = 0.25 Gauss. Spectra represent 100 Gauss and were normalized to the same spin concentration.

3.5. *Elucidation of the type of motion observed by ST-EPR*

The estimated rotational correlation times obtained for the OSL-Na,K-ATPase were almost an order of magnitude longer than expected at temperatures below 20°C (compared to an estimated rotational correlation time of 25 μs for the maleimide spin-labelled Na,K-ATPase at 0°C measured by Esmann and coworkers [17] and an estimated rotational correlation time of 60 μs at 4°C for the maleimide spin-labelled Ca-ATPase measured by Thomas and Hidalgo [23]). Since the long correlation times could indicate either membrane fragment rotation or large scale ATPase aggregate rotation as opposed to protomeric or diprotomeric ATPase rotation, we used selective glutaraldehyde crosslinking to elucidate which rotation mechanism was being observed [23].

OSL-labelled Na,K-ATPase membranes were incubated with glutaraldehyde briefly at a high protein

Table 2

Conventional and ST-EPR spectral parameters for glutaraldehyde crosslinked OSL-Na,K-ATPase membranes at 20°C

Sample parameter	Control membranes	Membrane fragment crosslinked	Protein-protein crosslinked
$2T_\parallel'$ (Gauss)	74.9	75.4	75.0
Δh_L (Gauss)	3.2 ± 0.2	3.0 ± 0.2	3.2 ± 0.2
% Weakly immobilized probes	0.8	0	0
$\int V_2'$	0.56 ± 0.07	0.75 ± 0.05	0.65 ± 0.05
τ_r ($\int V_2'$, μs)	45 ± 5	220 ± 10	100 ± 5
S_i'	0.56 ± 0.01	0.78 ± 0.05	0.63 ± 0.02
τ_r (S_i', μs)	45 ± 5	180 ± 15	85 ± 10

concentration (~ 50 mg/ml) for 10 min, an incubation designed to maximize crosslinking between membrane fragments and minimize crosslinking between proteins in the same membrane fragment. Alternatively, OSL-labelled Na,K-ATPase membranes were incubated with glutaraldehyde for 12 h at a low protein concentration (~1 mg/ml), an incubation designed to maximize crosslinking between adjacent proteins in individual fragments as opposed to crosslinking whole fragments. The samples were analyzed by electron microscopy to insure that each sample displayed the desired effect (data not shown).

Conventional and ST-EPR spectra of the OSL-Na,K-ATPase membranes crosslinked by each method are shown in fig. 5. The conventional spectral parameters $2T_{\parallel}'$, Δh_L and mole percent W of the crosslinked samples were similar to the spectra of control, non-crosslinked samples, indicating that the local environment of nitroxide had not changed upon crosslinking. ST-EPR analysis of the crosslinked samples showed that crosslinking between membrane fragments gave estimated τ_r values much longer (roughly a 4- to 5-fold increase) than the control values (i.e. $\tau_r = 220 \pm 10\,\mu s$ ($\int V_2'$) and $180 \pm 15\,\mu s$ (S_i') for the crosslinked fragments vs $44\,\mu s$ for the control) indicating that membrane fragment rotation was a principal mode of rotation (table 2). However, the protein-protein crosslinked samples also produced a rotational correlation time ($\tau_r = 85 \pm 10\,\mu s$) roughly two-fold longer than the control value ($\tau_r = 45 \pm 5\,\mu s$). The results suggest that the OSL-Na,K-ATPase polypeptides are rotating primarily as membrane fragments (or large scale aggregates within the fragment) yet contain a motional component from individual protein rotation in the order of (or slower than) the conglomerate rotation.

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