Effects of Ouabain on the Rotational Dynamics of Renal Na,K-ATPase Studied by Saturation-Transfer EPR†

James E. Mahaney and Charles M. Grisham*
Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901
Received August 9, 1991; Revised Manuscript Received November 19, 1991

ABSTRACT: The interaction of a nitroxide spin-labeled derivative of ouabain with sheep kidney Na,K-ATPase and the motional behavior of the ouabain spin label–Na,K-ATPase complex have been studied by means of electron paramagnetic resonance (EPR) and saturation-transfer EPR (ST-EPR). Spin-labeled 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 mole of ATP-dependent phosphorylation sites, even after repeated centrifugation and resuspension of the purified ATPase-containing membrane fragments. The conventional EPR spectrum of the ouabain spin label bound to the ATPase consists almost entirely (>99%) of a broad resonance at 0 °C, characteristic of a tightly bound spin label which is strongly immobilized by the protein backbone. Saturation-transfer EPR measurements of the spin-labeled ATPase preparations yield effective correlation times for the bound labels significantly longer than 100 μs at 0 °C. Since the conventional EPR measurements of the ouabain spin-labeled Na,K-ATPase indicated the label was strongly immobilized, 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 of ouabain spin-labeled Na,K-ATPase (a) cross-linked with glutaraldehyde and (b) crystallized in two-dimensional arrays indicated that the observed rotational correlation times predominantly represented the motion of large Na,K-ATPase-containing membrane fragments, as opposed to the motion of individual monomeric or dimeric polypeptides within the membrane fragment. The results suggest that the binding of spin-labeled ouabain to the ATPase induces the protein to form large aggregates, implying that cardiac glycoside induced enzyme aggregation may play a role in the mechanism of action of the cardiac glycosides in inhibiting the Na,K-ATPase.

Sodium and potassium ion activated adenosinetriphosphate (Na,K-ATPase)[1] (EC 3.6.1.3, ATP phosphohydrolase) is a plasma membrane transport system which is essential for normal cell function in all mammalian cells. The enzyme serves to maintain the high potassium and low sodium concentrations inside the cell that are fundamental in the control of cell volume and the electrical excitability of nerve and muscle cells (MacKnight & Leaf, 1977; Thomas, 1972). In addition, the Na,K-ATPase is acknowledged to be the primary pharmacologic receptor for cardiac glycosides (Hansen, 1983, 1984), a class of steroid-based drugs which are widely used in the treatment of congestive heart failure. Many aspects of the interaction between the Na,K-ATPase and cardiac glycosides (e.g., ouabain) have been studied, including the structure of the cardiac glycoside binding site on the enzyme [reviewed by Lingrel et al., (1990)] and the kinetics and affinity of glycoside binding [reviewed by Forbush (1983) and Hansen (1983, 1984)]. Clearly, understanding the interaction of the cardiac glycosides with the Na,K-ATPase and their physiological mechanism of action is important for improving the efficacy of their use in therapy while reducing their toxicity.

Ouabain analogues have proven to be important tools for structural studies of the cardiac glycoside binding site. The fluorescent analogue anthroylouabain (Fortes, 1977) has provided valuable information concerning the ouabain-bound enzyme conformation and binding site polarity (Fortes, 1977) and the spatial relationship between the ouabain binding site on the α-subunit and β-subunit oligosaccharides (Lee & Fortes, 1986). Photoaffinity analogues have extended this understanding by providing information about which subunits, and peptide domains on these subunits, compose the ouabain binding site [reviewed by Forbush (1983)].

While much effort has been devoted to understanding cardiac glycoside–Na,K-ATPase interactions, there is a more basic issue involved in the study of these interactions. Aside from potassium ions, the cardiac glycosides are the only specific agents which bind with high affinity to the extracellular surface of the Na,K-ATPase. In our laboratory, we have employed a variety of magnetic resonance and kinetic techniques to characterize the structure and arrangement of a variety of ATPase substrates, ion activators, and analogues on the enzyme surface (Grisham et al., 1974; Grisham & Hutton, 1978; O'Conner & Grisham, 1979, 1980; Gantzer et al., 1982; Klevickis & Grisham, 1982; Stewart & Grisham, 1988; Stewart et al., 1989). To date, however, all of our studies have involved molecules and ions which bind specifically to the

† This work was supported by National Institutes of Health Research Grant DK19419, a grant from the Muscular Dystrophy Association of America, and a grant from the National Science Foundation. The EPR instrumentation used in these studies was provided by grants from the National Science Foundation and the University of Virginia. C.M.G. is a Research Career Development Awardee of the U.S. Public Health Service (Grant AM00613).

* Author to whom correspondence should be addressed.

[1] Abbreviations: Na,K-ATPase, sodium and potassium ion activated adenosinetriphosphatase; OSL, ouabain spin label; OSL-Na,K-ATPase, ouabain spin-labeled Na,K-ATPase; MSL, maleimide spin label [N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide]; 4-amino-TEMPO, 2,2,6,6-tetramethyl-4-aminopiperidine 1-oxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris (TRIZMA), tris(hydroxymethyl)aminomethane; ATP, adenosine 5′-triphosphate; EPR, electron spin resonance; ST-EPR, saturation-transfer EPR; σ, ST-EPR V_1-integrated intensity parameter; $\int V_2'$, ST-EPR V_2'-integrated intensity parameter; TPX, tetramethylene polymer plastic.

0006-2960/92/0431-2025$03.00/0

intracellular surface of the ATPase. An analogous paramagnetic probe localized on the extracellular surface of the Na,K-ATPase would be an important tool for mapping the potassium sites and other relevant loci on the extracellular surface of the enzyme.

Solomonson and Barber (1984) have described the synthesis of a nitroxide derivative of ouabain and presented the results of some preliminary electron paramagnetic resonance (EPR) experiments. This spin label is the first paramagnetic probe known to bind with high affinity specifically to the extracellular face of the Na,K-ATPase, and, as a paramagnetic probe, this label offers a unique opportunity for locating and mapping the high-affinity K^+ binding site(s) on the extracellular face of the enzyme relative to the ouabain binding site using NMR relaxation measurements (Mildvan & Engle, 1972). However, reports of the use of this spin label in such studies or in more detailed EPR studies have not appeared in the literature.

In the present work, we describe a series of conventional EPR studies designed to better characterize the interaction of the ouabain spin label with renal NA,K-ATPase and a series of saturation-transfer EPR (ST-EPR) studies designed to elucidate the effects of ouabain on the rotational dynamics of the enzyme. Our results indicate that (a) the nitroxide moiety of the ouabain spin label is strongly immobilized by the protein backbone, making the label an excellent probe for studying the extracellular face of the Na,K-ATPase, and (b) the ouabain-inactivated enzyme is extensively aggregated in the membrane, providing new insight into the mechanism of action of cardiac glycosides against the Na,K-ATPase.

Materials and Methods

Reagents and Solutions. Ouabain octahydrate, sodium cyanoborohydride, sodium periodate, sodium phosphate, TRIZMA hydrochloride, imidazole, ATP, glutaraldehyde, and hemoglobin were purchased from Sigma Chemical Co. 4-Amino-TEMPO and maleimide spin label were purchased from Aldrich Chemical Co. Spin-labeled ouabain was synthesized according to Solomonson and Barber (1984). All other chemicals used were of the highest grade commercially available. All experiments were performed using buffer containing 30 mM MOPS, pH 7.2, and either 5 mM $MgCl_2$, 150 mM KCl, or 150 mM NaCl. Crystal samples were prepared in a buffer containing 1 mM sodium orthovanadate, 5 mM $MgCl_2$, and 20 mM Tris, pH 7.2. Buffers and appropriate reagent solutions were further purified by column treatment with Chelex-100 (Bio-Rad) to remove metal contaminants.

Enzyme Preparation and Assay. Na,K-ATPase was purified as membrane fragments from microsomal fractions of fresh sheep kidney outer medulla according to Jørgensen (1974) with modifications as described by O'Connor and Grisham (1979). Kidneys were obtained from freshly exsanguinated sheep at Rocco Further Processing, Inc., Timberville, VA, frozen in dry ice, and stored at −20 °C until use. Purified Na,K-ATPase was suspended in 10% (w/v) sucrose, pH 7.2, and stored at −20 °C until use. ATPase activity was measured using a coupled spectrophotometric assay at 37 °C according to Barnett (1970). Protein concentration was determined according to Lowry et al. (1951) using BSA as a standard. Enzyme used in these studies typically had a specific activity of 22–35 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}.

Spin Labeling Na,K-ATPase with Spin-Labeled Ouabain. Ouabain binds with highest affinity to the phosphorylated enzyme (Fortes, 1977; Hansen, 1983; Askari et al., 1988). Therefore, the Na,K-ATPase (2.5–3 mg/mL) was incubated with 1 mM spin-labeled ouabain in 10 mM MOPS, pH 7.2, containing 3 mM ATP, 3 mM $MgCl_2$, and 120 mM NaCl for 1 h at room temperature. Binding of the OSL was considered complete when the ouabain-sensitive ATPase activity had been completely abolished, as determined by enzyme-coupled continuous assay (Barnett, 1970). Unbound spin label was separated from the spin-labeled Na,K-ATPase by washing the membranes 3 times with 30 mL of the experimental buffer, pH 7.2, centrifuging each time at 25000*g* for 35 min. Following the final wash to remove unbound label, the sample was prepared immediately for EPR analysis as follows. The pellet was resuspended in 150 μL of experimental buffer, pH 7.2, and the membranes were centrifuged in a Beckman Airfuge at maximum speed for 10 min. Excess buffer was removed, and 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 (1981). Typical sample volumes were 30 μL.

Cross-Linking OSL-Na,K-ATPase with Glutaraldehyde. Glutaraldehyde cross-linking of spin-labeled Na,K-ATPase was performed using only fresh glutaraldehyde, and was performed according to Thomas and Hidalgo (1978). Spin-labeled Na,K-ATPase was prepared as above, then washed, and resuspended in 20 mM MOPS, pH 7.0. To maximize interfragment (i.e., fragment–fragment) cross-linking while minimizing intrafragment (i.e., protein–protein within each fragment) cross–linking, the protein was resuspended to 50 mg/mL and allowed to react with 1% glutaraldehyde for 15 min at room temperature. To maximize intrafragment cross-linking while minimizing interfragment cross-linking, the protein was resuspended to 1 mg/mL and allowed to react with 1% glutaraldehyde for 12 h at 4 °C. In each case, the cross-linking reaction was quenched by the addition of a large volume of 25 mM MOPS, pH 7.0, and the cross-linked membranes were washed twice in 20 mM MOPS, pH 7.0, to remove unreacted glutaraldehyde. The samples were prepared for EPR analysis as described above. The extent of cross-linking by each protocol was confirmed by electron microscopy of negatively stained preparations according to Manella (1982), which showed pronounced aggregation of fragments in the interfragment cross-linked sample and no significant aggregation in the intrafragment cross-linked sample.

Preparation of Two-Dimensional Crystals of OSL-Na,K-ATPase. 2D crystals of OSL-Na,K-ATPase were prepared by simultaneously spin labeling and crystallizing the enzyme. The method for crystallizing the Na,K-ATPase was adapted from the method of Skriver et al. (1981), using 5 mM $MgCl_2$ and 1 mM orthovanadate. Orthovanadate solutions were prepared according to Goodno (1982). Na,K-ATPase (3 mg/mL) was incubated with 1 mM OSL in 3 mM ATP, 120 mM NaCl, 5 mM $MgCl_2$, and 1 mM orthovanadate in 20 mM Tris-HCl, pH 7.2. The suspension was allowed to incubate for more than 48 h at 4 °C, followed by three washes in 1 mM orthovanadate and 5 mM $MgCl_2$ in 10 mM Tris-HCl, pH 7.2, to remove unbound label. Samples were prepared for EPR as described above. The extent of crystallization in the samples was determined in separate experiments by electron microscopy of negatively stained preparations according to Manella (1982).

Solubilization of OSL-Na,K-ATPase with $C_{12}E_8$. $C_{12}E_8$-solubilized samples of OSL-Na,K-ATPase were prepared according to Esmann and Skou (1984). The membrane-bound enzyme was spin labeled with OSL and washed as described above, followed by a final centrifugation at 100000 rpm for 10 min in a Beckman Airfuge. The supernatant was discarded, and the pellet was resuspended using

80 μL of 25% glycerol (v/v) in 20 mM imidazole, pH 7.2, with final concentrations typically 10–20 mg/mL. To 9 volumes of the ice-cold protein was added 1 volume of ice-cold $C_{12}E_8$ in water, under vortex, to a final detergent:protein ratio of 3:1. The mixture was immediately centrifuged at 100000*g* for 10 min in an Airfuge to remove the nonsolubilized residue. In preparation for EPR analysis, the solubilized, spin-labeled enzyme was concentrated to 20–30 mg/mL in an Amicon Centricon 30 microconcentrator and then drawn directly into the TPX sample capillary by gentle suction, in preparation for EPR analysis.

EPR Spectroscopy. 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 E231 cavity dewar insert equipped with a heater–sensor probe. Spectra were digitized as 1000 points and stored on floppy disks with an AT&T 6300 PC using an IBM compatible software/interface package developed by Morse (1987). Conventional (V_1) EPR spectra were recorded using 100-kHz modulation, with a peak-to-peak amplitude of 2 G, and microwave field intensities (H_1) of either 0.032 or 0.25 G. Saturation-transfer (V_2') spectra were recorded using 50-kHz modulation, with a peak-to-peak amplitude of 5 G, 100-kHz detection set 90° out-of-phase, and a microwave field intensity of 0.25 G. All spectra were recorded using a 100-G sweep width. Adjustment of the out-of-phase detection condition for V_2' spectral acquisition was done by the self-null method, and appropriate selection and determination of H_1 were done by comparison with peroxylaminedisulfonate, both as described by Squier and Thomas (1986a). All measurements were performed in the absence of oxygen, which was removed from the samples via the gas-permeable TPX sample capillary (Popp & Hyde, 1981) described above. 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 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 for sample equilibration and to allow removal of dissolved molecular oxygen (Popp & Hyde, 1981). Neither the shim nor the TPX holder extended into the cavity, which thus contained only the sample in the TPX tubing.

EPR Spectral Analysis. The spin concentration in OSL-Na,K-ATPase samples was determined by comparison of the double integral of the experimental V_1 spectra, recorded at low power (H_1 = 0.032 G), to the double-integral values obtained from spectra of 0.1, 0.2, and 0.4 mM OSL standards, recorded and integrated in an identical manner. All spectra are shown normalized to the same number of spins (except where noted), achieved by dividing each V_1 spectrum by a number proportional to its double integral, or by dividing each V_2' spectrum by a number proportional to the double integral of its corresponding V_1 spectrum.

The rotational mobility of the OSL-Na,K-ATPase was studied using the integrated intensity parameter, denoted σ (Squier & Thomas, 1989), which is obtained from V_1 spectra collected at low, nonsaturating power (LP, H_1 = 0.032 G) and at high, saturating power (HP, H_1 = 0.25 G) for each sample according to Squier and Thomas (1986a):

$$\sigma = \left\{ \frac{\left[\int\int V_1/H_1\right]_{LP}}{\left[\int\int V_1/H_1\right]_{HP}} \right\} - 1 \qquad (1)$$

As a complementary measure of OSL-Na,K-ATPase rotational mobility, intensity parameters derived from V_2' spectra, denoted V_2'IP, were also obtained, using the integrals of V_2' spectra normalized to the double integral of their corresponding V_1 spectra according to Squier and Thomas (1986a):

$$V_2'\text{IP} = \frac{\int V_2'}{\left[\int\int V_1/H_1\right]_{LP}}$$

Rotational correlation times (τ_r) of the enzyme were determined by comparing the derived intensity parameters to standard curves constructed from an isotropically tumbling model system, described fully by Squier and Thomas (1986a) and Thomas et al. (1976). For every sample, the effective τ_r values obtained by the two parameters displayed good agreement. It is important to note that since τ_r values are derived from an isotropically rotating system, these values can only be considered as "effective" correlation times describing the rotation of the OSL-Na,K-ATPase, whose rotation is predicted to be anisotropic within its membrane system (Saffman & Delbrück, 1975). Nevertheless, effective τ_r values provide a convenient tool for comparing OSL-Na,K-ATPase rotation under the various conditions employed in this study.

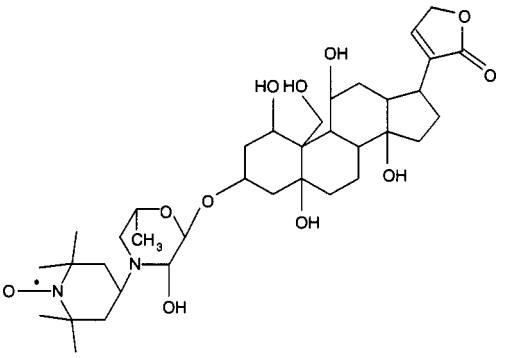

FIGURE 1: Structure of the ouabain spin label (OSL) used in this study.

RESULTS

Characterization of Ouabain Spin Label Binding. The ouabain spin label (OSL, Figure 1) used in these experiments was synthesized according to Solomonson and Barber (1984), and the structure was confirmed by mass spectrometric analysis. As previously reported (Solomonson & Barber, 1984), 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 enzymic activity. Solomonson and Barber's original report did not describe the stoichiometry of binding of the ouabain spin label to the Na,K-ATPase. Therefore, we compared the integrated intensity of OSL-Na,K-ATPase spectra to the integrated intensities of a series of free ouabain spin label standard spectra. From this comparison, a binding stoichiometry of 0.61 ± 0.1 mol of spin label bound per mole of ATP-dependent phosphorylation sites was calculated, consistent with results obtained with normal ouabain (Askari et al., 1988).

Conventional EPR Analysis of OSL-Na,K-ATPase. A typical spectrum of the ouabain spin label bound to the Na,K-ATPase (OSL-Na,K-ATPase) suspended in 30 mM MOPS, pH 7.2, is shown in Figure 2 (inset, initial). Consistent with the results of Solomonson and Barber (1984), the spectrum is composed of a predominantly broad resonance, typical of a population of highly immobilized spin labels ($\tau_r \geq 10^{-6}$ s), and a minor narrow resonance, typical of a population of weakly immobilized nitroxides ($\tau_r \approx 10^{-9}$ s). The narrow resonance could not be removed by longer incubation times

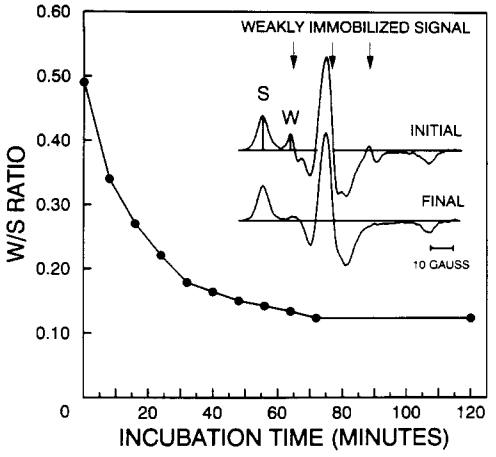

FIGURE 2: Time-dependent loss of weakly immobilized signal during 0 °C incubation. Initially (time = 0 min), OSL-Na,K-ATPase spectra contained a weakly immobilized spectral component denoted W (inset, initial), which diminished nearly exponentially (fit not shown) during an hour-long incubation at 0 °C to a level indistinguishable from the strongly immobilized signal (inset, final), after which the spectrum remained constant when held at 0 °C. Inset spectra are OSL-Na,K-ATPase in 30 mM MOPS, pH 7.2, and represent 100 G.

during spin labeling or other alterations of the incubation conditions, nor was it removed by further centrifugation and washing of the spin-labeled enzyme pellet. Deconvolution of the spectrum by computer spectral subtraction produced two single-component spectra (data not shown). From the subtractions, as verified by double integration, the mole fraction of the weakly immobilized component was determined to be 0.03 ± 0.003.

Time-Dependent Loss of the Weakly Immobilized Component. Although the weakly immobilized spectral component could not be eliminated by variation of spin-labeling conditions, time of incubation, or washing, the contribution of the weak component did decrease with time, as shown in Figure 2. Within a period of 60–72 min at 0 °C, the mole fraction of the weakly immobilized component decreased nearly exponentially from the initial mole fraction of 0.03 ± 0.003 (corresponding to a W/S ratio of 0.49) to a level of 0.005 ± 0.001 (W/S = 0.12), leaving a spectrum which was indistinguishable from a single-component, strongly immobilized spectrum (Figure 2 inset, final). The loss of the weakly immobilized component was accompanied by an overall loss of spectral intensity of 2.9 ± 0.2%, after which the spectrum remained constant at 0 °C, as quantified by double integration, despite longer incubations. This behavior was observed for all freshly spin-labeled samples; thus, all EPR samples were incubated for 60 min at 0 °C prior to spectral acquisition.

Dependence of Spin-Labeled ATPase Spectra on Ionic Environment. The Na,K-ATPase is known to assume distinct conformations in the presence of different physiological ligands (Jørgensen et al., 1982; Hegyvary & Jørgensen, 1981; Esmann, 1987). To determine if the conventional (V_1) spectrum of OSL-Na,K-ATPase is sensitive to the physiological ligands Mg^{2+}, K^+, and Na^+, spectra were collected with the spin-labeled ATPase suspended in media containing either 5 mM $MgCl_2$ (denoted Mg^{2+} environment), 150 mM KCl (denoted K^+ environment), or 150 mM NaCl (denoted Na^+ environment), as shown in Figure 3A–C (left column). The effect of ATP on the OSL-Na,K-ATPase spectrum was not studied, since ATP is known to promote dissociation of ouabain (hence the spin label) from the enzyme (Forbush, 1983; Hansen, 1984; Askari et al., 1988). The spectrum at 0 °C for each ionic environment is nearly identical with respect to the maximum

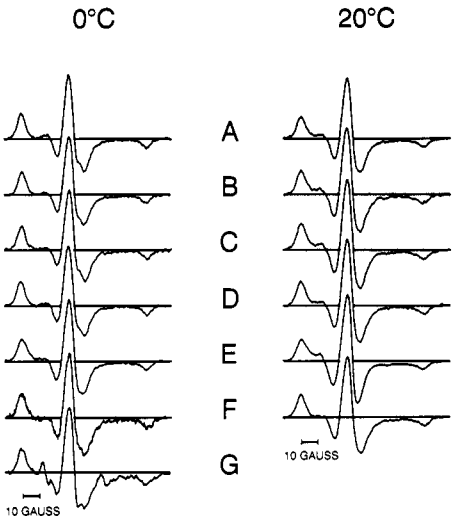

FIGURE 3: Conventional EPR spectra of OSL-Na,K-ATPase at 0 and 20 °C in 30 mM MOPS, pH 7.2, either suspended with (A) 5 mM $MgCl_2$, (B) 150 mM NaCl, or (C) 150 mM KCl, or glutaraldehyde cross-linked providing (D) cross-linked membrane fragments or (E) cross-linked proteins within membrane fragments, or (F) crystallized with 5 mM $MgCl_2$ and 1 mM orthovanadate, or (G) solubilized with $C_{12}E_8$. Spectra were characterized by the outer splitting ($2T_\parallel'$), the outer half-width at half-height of the low-field peak (Δ_L), both in Gauss, and the presence of weakly immobilized probes (W/S), as defined in Figure 4. Spectra are shown normalized to the same gain and spin concentration, and represent 100 G.

hyperfine splitting, $2T_\parallel'$, the half-width at half-height of the low-field peak, Δ_L, and the W/S ratio, which qualitatively reports the changing mole fraction of the weakly immobilized component. The consistency of these parameters in the presence of each ligand suggests that there is no ion-dependent change in the local environment of the spin label on the protein backbone.

Temperature Dependence of Spin-Labeled Na,K-ATPase Spectra. The conventional EPR spectra of the OSL-Na,K-ATPase suspended in each ionic environment at 20 °C are shown in Figure 3A–C (right column). For each ligand, the conventional spectral parameters were clearly temperature dependent (Figure 4). At 0 °C, the strongly immobilized component was dominant (>99% of total spectral intensity), and $2T_\parallel'$ was at a maximum, whereas Δ_L and the W/S ratio were at a minimum. With increasing temperature, $2T_\parallel'$ decreased (Figure 4A) while Δ_L (Figure 4B) and the W/S ratio (Figure 4C) increased. Together, the increasing $2T_\parallel'$ and decreasing Δ_L indicated faster motion of the spin label and/or label–protein complex and the increasing W/S ratio indicated the return of a minor component of weakly immobilized probes. When the sample temperature was returned to 0 °C, both $2T_\parallel'$ and Δ_L returned to the original values, but the mole fraction of the weak component had increased slightly (not shown). The irreversible temperature behavior of the weakly immobilized probes may have resulted from either (a) a loosening of a small fraction of bound labels (i.e., the nitroxide moieties of the bound molecules) within their binding site or (b) a small fraction of the labels dissociating from the protein, giving rise to a small free label spectrum convoluted into the bound label spectrum.

Temperature-Dependent Loss of Spectral Intensity. For each OSL-Na,K-ATPase sample studied, we observed the initial time-dependent loss of the weakly immobilized component during the hour-long 0 °C incubation, and once stabilized, the intensity of each spectrum remained constant as long as the sample was held at 0 °C (described above). However, as each sample was warmed to 37 °C, the spectral

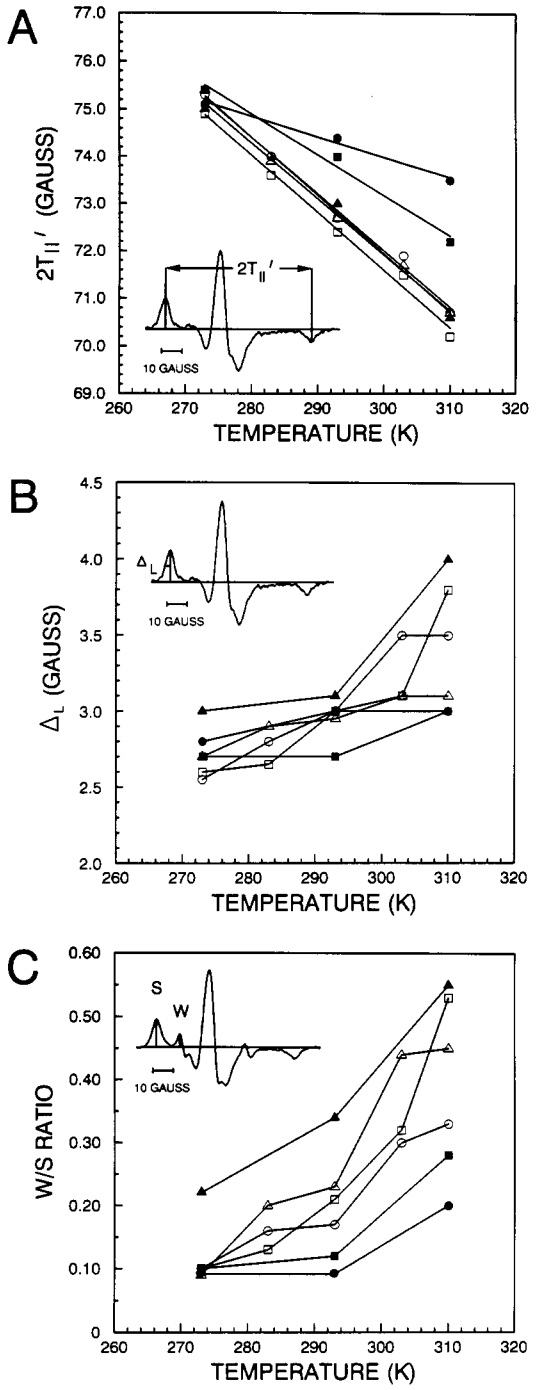

FIGURE 4: Temperature dependence of the conventional EPR spectral parameters $2T_{\parallel}'$ (A), Δ_L (B), and W/S (C) for OSL-Na,K-ATPase in 30 mM MOPS, pH 7, under the following conditions: 150 mM NaCl (open boxes), 150 mM KCl (open triangles), 5 mM $MgCl_2$ (open circles), glutaraldehyde cross-linked membrane fragments (closed boxes), glutaraldehyde cross-linked proteins within membrane fragments (closed triangles), crystallized with 5 mM $MgCl_2$ and 1 mM orthovanadate (closed circles). The data in (A) are shown fit to least-squares lines. Typical standard deviations for the parameters were the following: $2T_{\parallel}'$, ±0.3 G; Δ_L, ±0.1 G; W/S, ±0.2, and were obtained from at least two measurements.

intensity steadily decreased. Comparison of the total spectral intensity present during the first acquisition at 0 °C to that of an identical 0 °C acquisition after cycling a given sample to 37 °C indicated that on average, more than 50% of the total spectral intensity was lost as a function of warming. The most likely source of spectral hysteresis, temperature-induced reduction of the spin label while bound to the protein, is considered below (see Discussion).

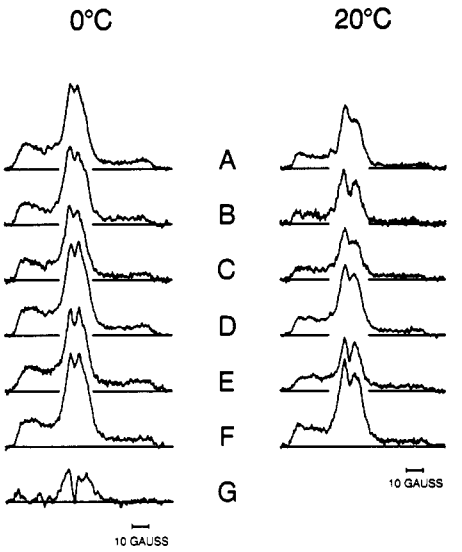

FIGURE 5: V_2' ST-EPR spectra of OSL-Na,K-ATPase at 0 and 20 °C, as defined in Figure 3. Spectra are shown normalized to the same gain and spin concentration and represent 100 G.

ST-EPR Spectral Analysis of OSL-Na,K-ATPase: Effect of Temperature and Ionic Environment. We used saturation-transfer EPR (ST-EPR) to characterize the nature of ouabain-inactivated Na,K-ATPase rotation and to determine if there exists functionally relevant changes in this rotation as a function of ligand environment (Bentley, 1983; Esmann et al., 1987). ST-EPR (V_2') spectra of the OSL bound to the Na,K-ATPase at 0 and 20 °C in Mg^{2+}, K^+, and Na^+ environments are shown in Figure 5A–C. The spectra in each ionic environment contain marked changes in line-shape detail and total intensity in the 20 °C spectrum versus the 0 °C spectrum, typical of increased rotational mobility of the label–protein complex at the higher temperature (Squier & Thomas, 1986a). Still, at both temperatures, the V_2' spectra were characteristic of a highly immobilized spin-label–protein complex. Low signal-to-noise in the V_2' spectra prevented accurate V_2' spectral analysis by the common line-height ratio method (Squier & Thomas, 1986a; Thomas et al., 1976), and the temperature-dependent spectral hysteresis (described above) rapidly diminished the utility of the V_2' intensity parameter (Squier & Thomas, 1986a) with increasing temperature. The method of choice for ST-EPR analysis in this study, therefore, was the in-phase V_1 intensity parameter, denoted σ (Squier & Thomas, 1989). Whereas intensity parameters were calculated whenever possible from V_2' spectra, these parameters were used only as a complementary measure of τ_r.

In-phase intensity parameters and effective rotational correlation times for the spin-labeled protein were obtained from spectra of the OSL-Na,K-ATPase in each ionic environment studied (Mg^{2+}, Na^+, and K^+) at temperatures ranging from 0 to 37 °C, including an additional measurement after returning the sample to 0 °C. The result of this analysis is presented in Figure 6 and Table I. The derived σ intensity parameters and corresponding effective τ_r values for K^+ and Na^+ environments were fairly similar at all temperatures (except 0 °C), while those values for the Mg^{2+} environment were distinctly different. These σ values yielded effective τ_r values of 399 ± 17 μs for the Mg^{2+} environment at 0 °C and 191 ± 10 and 208 ± 11 μs for the K^+ and Na^+ environments at 0 °C, respectively. With increasing temperature, the σ values decreased, consistent with faster OSL-Na,K-ATPase rotational motion. Upon warming to 37 °C, the effective

Table I: Effects of Ionic Environment and Aggregation State on the Effective Rotational Correlation Time (μs) of OSL-Na,K-ATPase[a]

temp (°C)	Mg^{2+}	K^+	Na^+	protein cross-linked	fragment cross-linked	2D crystals
0	399 ± 21	191 ± 10	208 ± 11	170 ± 12	300 ± 17	525 ± 20
10	161 ± 11	81 ± 5	109 ± 8			
20	90 ± 9	44 ± 6	45 ± 4	85 ± 9	180 ± 15	214 ± 10
30	70 ± 4	30 ± 2	30 ± 2			
37	44 ± 2	20 ± 2	21 ± 1	56 ± 7	110 ± 10	100 ± 11
0 RPT	189 ± 15	112 ± 9	112 ± 9	120 ± 12	200 ± 15	500 ± 25

[a] OSL-Na,K-ATPase effective rotational correlation times were determined using the V_1 integrated intensity parameter, σ, and a standard curve constructed from an isotropically tumbling model system as described under Materials and Methods. Mg^{2+}, K^+, and Na^+ correspond to sample buffers containing 30 mM MOPS, pH 7.2, and either 5 mM $MgCl_2$, 150 mM KCl, or 150 mM NaCl, respectively. Protein and fragment cross-linked correspond to spin-labeled samples in 30 mM MOPS, pH 7.2, which were treated with glutaraldehyde to preferentially cross-link proteins within individual membrane fragments versus those in separate membrane fragments, respectively. 2D crystals corresponds to spin-labeled enzyme in membranous 2D crystal aggregates in 20 mM Tris, pH 7.2, which were formed in the presence of 5 mM $MgCl_2$ and 1 mM orthovanadate. 0 RPT corresponds to measurements made on samples at 0 °C after warming to 37 °C. Sample preparations are described under Materials and Methods. Errors represent the standard deviation of at least two measurements.

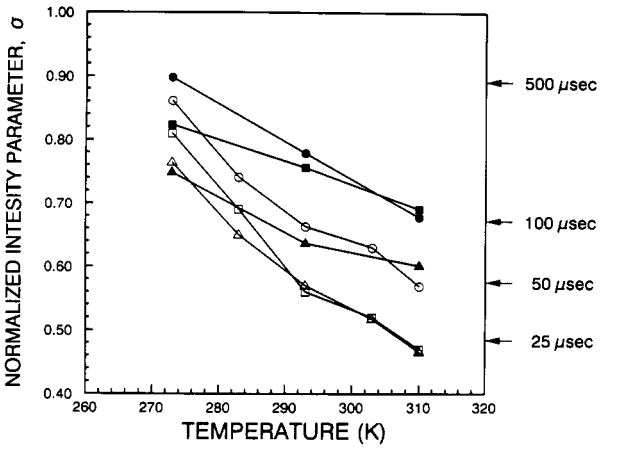

FIGURE 6: Temperature dependence of the in-phase saturation-transfer intensity parameter, σ, measured for OSL-Na,K-ATPase under the conditions defined in Figure 4. The effective rotational correlation times corresponding to these intensity parameters (Table I) were deduced from standard curves constructed using an isotropically tumbling model system (see Materials and Methods).

correlation times had decreased to 44 ± 5 μs for the Mg^{2+} environment and 20 ± 2 and 21 ± 2 μs for the K^+ and Na^+ environments, respectively. Intensity parameters derived from the V_2' spectra gave effective rotational correlation times which were consistent with those derived from the σ parameter.

Elucidation of the Type of Motion Observed by ST-EPR. To identify whether the rotational motion observed corresponded to rotation of the OSL-Na,K-ATPase itself or overall rotation of the OSL-Na,K-ATPase-containing membrane fragments, we used selective glutaraldehyde cross-linking procedures similar to those described by Thomas and Hidalgo (1978). In one of these procedures, the OSL-Na,K-ATPase membranes were incubated with glutaraldehyde briefly at a high protein concentration (≈50 mg/mL) for 10 min, an incubation designed to maximize cross-linking between proteins in different membrane fragments and minimize cross-linking between proteins within the same membrane fragment. Alternatively, OSL-Na,K-ATPase membranes were incubated with glutaraldehyde for 12 h at a low protein concentration (≈1 mg/mL), an incubation designed to maximize cross-linking between proteins in individual fragments, as opposed to cross-linking proteins in different fragments. The samples prepared in each cross-linking experiment were analyzed by electron microscopy (data not shown) to confirm that each sample displayed the desired effect (i.e., that the brief cross-linking produced aggregate membrane fragments and that the long cross-linking produced no significant change in membrane fragment aggregation). V_1 and V_2' spectra of the OSL-Na,K-ATPase membranes cross-linked by each method are shown in Figures 3D,E and 5D,E, respectively. The conventional EPR parameters $2T_\parallel'$, Δ_L, and W/S were similar to those obtained for OSL-Na,K-ATPase in Mg^{2+}, K^+, and Na^+ solutions, indicating that the local environment of the nitroxide in each cross-linked sample was also similar. However, the consistently greater $2T_\parallel'$ values, together with the consistently smaller Δ_L and W/S values obtained from the fragment–fragment cross-linked sample, indicated that the rotational mobility of the OSL-Na,K-ATPase in this sample was more restricted than that of the untreated OSL-Na,K-ATPase either in the Mg^{2+}, K^+, or Na^+ environments or in the protein–protein cross-linked sample. This finding allowed the preliminary interpretation that the type of rotation exhibited by the OSL-Na,K-ATPase in the Mg^{2+}, K^+, or Na^+ environments was characteristic of overall membrane fragment rotation rather than rotation of individual OSL-Na,K-ATPase monomers, dimers, or oligomers within the fragments. Analysis of the σ intensity parameters obtained from the two types of cross-linked samples over the temperature range 0–37 °C indicated that cross-linking between membrane fragments gave effective τ_r values generally longer (with the exception of the 0 °C Mg^{2+} environment measurement) than the values obtained from untreated OSL-Na,K-ATPase in the presence of Mg^{2+}, Na^+, and K^+ whereas the correlation times obtained from the protein–protein cross-linked sample were similar to those obtained in the three ionic environments (Figure 6, Table I). This finding, which is consistent with conventional EPR data, indicates that the predominant motion observed was the overall rotation of the OSL-Na,K-ATPase as entire membrane fragments.

Formation of Two-Dimensional Membrane Crystals of OSL-Na,K-ATPase. Purified, membrane-bound Na,K-ATPase will form two-dimensional (2D) membrane crystals in the presence of Mg^{2+} and either phosphate or vanadate under conditions known to promote the E_2–P (or E_2–V) form of the enzyme (Skriver et al., 1981; Maunsbach et al., 1988). Since ouabain (and thus OSL) binding locks the protein in the E_2 (or an E_2-like) conformation (Forbush, 1983; Hansen, 1983, 1984), we sought to determine if the extremely slow rotation observed for the protein in the Mg^{2+} environment was reflecting the formation of an OSL-Na,K-ATPase aggregate similar either in size or in structure to 2D membrane crystals. We tested this hypothesis by forming (2D) membrane crystals with vanadate and Mg^{2+} in the presence of OSL. As before, the samples were analyzed by electron microscopy (data not shown) to ensure that each sample displayed the desired crystallization.

Typical V_1 and V_2' spectra for crystallized OSL-Na,K-ATPase are shown in Figures 3F and 5F. Spectral analysis of $2T_\parallel'$, Δ_L, and W/S indicated that, as in the other samples,

the local environment of the nitroxide was not perturbed by crystal formation (Figure 4). However, of all the data shown in Figure 4, the crystallized OSL-Na,K-ATPase sample generally displayed label with the greatest immobilization, suggesting the slowest rotational motion of all the samples over the temperature range of 0–37 °C. This was supported by σ intensity parameter analysis (Figure 6, Table I) which showed that the rotation of the crystallized OSL-Na,K-ATPase was substantially inhibited (525 ± 20 μs at 0 °C) relative to the other samples (e.g., roughly 125 μs slower than the protein rotation in a Mg^{2+} environment and roughly 325 μs slower than the Na^+ and K^+ environments). At every temperature studied, the crystallized OSL-Na,K-ATPase sample rotated more slowly than the Mg^{2+} environment sample, suggesting that the nature of the rotating OSL-Na,K-ATPase unit in the Mg^{2+} environment, though clearly an aggregate, does not fully approximate the nature of the crystallized OSL-Na,K-ATPase rotating unit. However, it remains clear that the nature of the rotating species in the Mg^{2+} environment is distinct from that of the rotating species in the Na^+ and K^+ environments.

Disruption of Large-Scale Protein Aggregation with $C_{12}E_8$. To characterize the rotational mobility of OSL-Na,K-ATPase in primarily protomeric units, the spin-labeled membranes were treated with $C_{12}E_8$ (Brotherus et al., 1983) as described under Materials and Methods. A V_1 spectrum of the solubilized OSL-Na,K-ATPase at 0 °C is shown in Figure 3G. The solubilized OSL-Na,K-ATPase samples typically contained only 60 μM spin label, and therefore required significant signal averaging to produce spectra with sufficient signal-to-noise ratios for analysis. Conventional EPR analysis indicated that $2T_\parallel'$ (74.6 G) was similar to the membrane-bound protein samples while Δ_L (3.1 G) was significantly larger than the membrane-bound samples, indicative of protein motional differences in the soluble form as compared to the membranous form. One prominent difference between the solubilized and membrane-bound samples, however, was the presence of a weakly immobilized signal in the spectrum (roughly 11% by deconvolution and double integration). This mobile population was characteristic of nanosecond label rotation and, in contrast to the membrane-bound OSL-Na,K-ATPase, was stable over several hours at 0 °C. Increasing sample temperature induced rapid signal intensity decreases, resulting in signal too weak to discern above the base-line noise despite very lengthy signal collection and averaging. Therefore, spectra were obtained only at 0 °C for the solubilized system.

The saturation-transfer V_2' spectrum of the $C_{12}E_8$-solubilized spin-labeled protein at 0 °C is shown in Figure 5G. Analysis of σ intensity parameters gave an effective τ_r for the system of 12 ± 1 μs at 0 °C (in good agreement with the $V_{2'}$ intensity parameter). Though the conditions used to solubilize the OSL-Na,K-ATPase are reported to produce predominantly monomeric protein (Brotherus et al., 1983; Esmann, 1987), the exact aggregation state of the solubilized unit (monomeric, dimeric, or higher oligomer) in our application was not determined. Regardless, the effective τ_r value for the solubilized protein at 0 °C is approximately 33-fold smaller than that of the membrane-bound protein in the Mg^{2+} environment and approximately 16 times faster than that of the membrane-bound protein in the Na^+ and K^+ environments, consistent with a significantly smaller rotating unit in the solubilized versus membrane-bound form.

Discussion

Summary. We have characterized the interaction between Na,K-ATPase and the ouabain spin label first reported by Solomonson and Barber (1984) using conventional EPR, and have used this spin label to study the rotational dynamics of the ouabain-inactivated Na,K-ATPase with ST-EPR. The results show that the label binds to the enzyme in a single, highly immobilized environment and that the ouabain-inactivated enzyme is highly aggregated. These results suggest that ouabain-facilitated Na,K-ATPase aggregation may serve to stabilize the ouabain-induced, ligand-resistant conformational state of the enzyme responsible for its inactivation.

OSL Is Strongly Immobilized When Bound to Na,K-ATPase. The conventional EPR spectra of OSL-Na,K-ATPase (Figure 3) show clearly that, regardless of the cationic environment (Mg^{2+}, K^+, or Na^+) or physical state (glutaraldehyde cross-linked or 2D crystal aggregates) of the membrane-bound enzyme, the ouabain spin label is bound to the Na,K-ATPase in a highly immobilized environment on the enzyme. At lower temperatures (0–10 °C), the spectra are indicative of label in a single environment, since the spectra contain no significant contribution from weakly immobilized probes. The outer splittings of the spectra ($2T_\parallel'$ in Figure 4A) at all temperatures between 0 and 37 °C are unusually large (>70 G) relative to values obtained for other spin-labeled Na,K-ATPase preparations (Esmann et al., 1987; 1989; 1990), suggesting either that (a) the environment of the OSL nitroxide is more polar than that of sulfhydryl-linked labels or that (b) the mobility of the OSL nitroxide itself relative to the enzyme is more restricted than the sulfhydryl-linked spin labels. We conclude the latter, since the Δ_L values for each OSL-Na,K-ATPase preparation between 0 and 20 °C are less than 3 G (Figure 4B), and the environment of the ouabain binding site has previously been shown to be hydrophobic and shielded from water (Fortes, 1977). Furthermore, it is possible that at 0 °C, the value of $2T_\parallel'$ (≈75 G) may be nearly equivalent to the spin-label's rigid limit value, since all OSL-Na,K-ATPase preparations, including the most mobile $C_{12}E_8$ sample and most immobile 2D crystalline sample, have similar values.

Temperature-Dependent Label Dissocation and Spectral Hysteresis. An intriguing aspect of the interaction between the spin label and the enzyme is the temperature-induced spectral hysteresis. The V_1 spectra of freshly prepared OSL-Na,K-ATPase initially contain a significant (≈3%) contribution from a second, more mobile population of spin labels (Figure 2 inset, initial), similar to the spectra of Solomonson and Barber (1984). Changes in binding conditions and washing procedures could not remove this initial mobile population, indicating that it does not represent ouabain spin label partitioned into the membrane fragment lipid or free spin label in the aqueous phase. Rather, this component probably represents a minor population of spin labels which were bound more weakly by the enzyme relative to the majority of tightly bound, highly immobilized labels. This is consistent with the proposal of Solomonson and Barber (1984), who suggested that these weakly bound labels reflect the distribution between bound and unbound forms of OSL at equilibrium.

The disappearance of the weakly immobilized signal (Figure 2) was accompanied by an equivalent loss of spectral intensity during the hour-long 0 °C incubation, and it remains unclear whether this loss of intensity was caused by (a) preferential reduction of the weakly bound labels or (b) nonspecific hysteresis of all labels as the weakly immobilized labels bound more tightly. Kinetic analyses of the ouabain–Na,K-ATPase interaction have shown clearly that increasing temperature increases the rate of dissociation of ouabain from the enzyme [reviewed by Forbush (1983) and Hansen (1984)], which is consistent with our observation that increasing sample tem-

perature induces increases in the fraction of weakly immobilized probes (Figure 4C). Since increasing temperature also promotes progressive spectral hysteresis, it appears the two processes may be correlated, implying that the environment of the weak probes facilitates nitroxide reduction whereas the nitroxide environment of the strongly immobilized probes protects this population of nitroxides against reduction. Rauckman et al. (1984) and Perussi et al. (1988) have shown that protein sulfhydryl groups can reduce nitroxides, and several studies have shown that there are free sulfhydryl groups on the extracellular face of both the α and β subunits of Na,K-ATPase, presumably in proximity to the ouabain binding site (Shull et al., 1985, 1986; Price & Lingrel, 1988; Kirley et al., 1986; Scheiner-Bobis et al., 1987). It is possible that a highly immobilized nitroxide cannot interact with any of these sulfhydryl groups, whereas a weakly immobilized nitroxide (or a dissociating label), which has the ability to tumble within its microenvironment, can. Further, given the unusually large $2T_{\parallel}'$ values (Figure 4A), it is suspected that the six-member nitroxide-bearing ring of the spin label bound to the enzyme is oriented in a boat conformation to facilitate a hydrogen bond between the nitroxide oxygen and the protonated amine of the OSL sugar moiety. This conformation would position the nitroxide N–O bond outside the protection of the adjacent methyl groups, leaving this bond (thus the intact unpaired electron) more susceptible to attack from any of the free sulfhydryl groups on the extracellular face of the protein. Future studies using a tritiated ouabain spin label should permit a quantitative test of this hypothesis.

Ouabain-Inactivated Na,K-ATPase Is Extensively Aggregated. Freeze–fracture electron microscopy studies have shown that, though the Na,K-ATPase is densely packed in its host membrane [estimated density of 1 g of protein/mL of lipid (Jørgensen, 1985)], the enzyme is randomly oriented both in vivo and when isolated as membrane fragments (Maunsbach et al., 1988). In contrast, our ST-EPR studies of OSL-Na,K-ATPase mobility indicate that the rotation of ouabain-inactivated enzyme is greatly restricted, with correlation times which are significantly longer than those expected for a monomeric or dimeric Na,K-ATPase unit in the membrane.

Comparison of the rotational correlation times determined in the present work with values determined for Na,K-ATPase in the absence of ouabain and for other membrane-bound enzymes lends support to the hypothesis of aggregation of OSL-labeled Na,K-ATPase. Rotational correlation times measured from ST-EPR intensity parameters for maleimide spin-labeled Na,K-ATPase (Esmann et al., 1987) are less than 1 μs at all temperatures examined. Using a benzoylvinyl spin label (which exhibits greatly reduced segmental mobility) to label sulfhydryl residues on Na,K-ATPase, Esmann et al. (1989) determined values of τ_r of 20 μs at 0 °C and 2 μs at 37 °C from ST-EPR intensity parameters. Recently, Esmann et al. (1990) used a series of similarly motionally restricted vinyl ketone spin labels to measure ATPase protein rotational diffusion. The average value of τ_r determined from 12 vinyl ketone spin labels with Na,K-ATPase was 64 μs at 0 °C. Even the longest of these values is significantly smaller than the τ_r values determined for the OSL-labeled Na,K-ATPase in the present work.

The rotational diffusion of other integral membrane proteins has also been examined by ST-EPR methods. Squier and Thomas (1986b) determined values of τ_r of 17 μs at 0 °C and 5 μs at 18 °C for maleimide spin-labeled SR Ca-ATPase, and Lewis and Thomas (1986) observed a value of τ_r for 2D crystalline SR Ca-ATPase of 23 μs. The latter value, however, was presumed to indicate significant segmental motion of the maleimide spin label in the crystalline ATPase. Several other groups have reported values of τ_r for integral membrane proteins using ST-EPR line-height ratios (which tend to be up to an order of magnitude longer than similar values obtained from intensity ratios). These include values of τ_r of 40 μs for beef heart cytochrome *c* oxidase (complex IV) (Swanson et al., 1980), 70 μs for beef heart cytochrome *c* reductase (complex III) (Quintanilha et al., 1982), and 20 μs for rhodopsin (Baroin et al., 1977). Interestingly, Schwarz et al. (1982) have reported a value of τ_r for rabbit liver cytochrome P-450 of 480 μs (from line-height ratios), stating that such a large value of τ_r must mean that the protein is highly aggregated in the membrane.

Additional support for our interpretation that OSL-labeled Na,K-ATPase is aggregated in the membrane is provided by our cross-linking and 2D crystal experiments (Figure 6). The large decrease in enzyme rotational mobility in the fragment–fragment cross-linking study indicated that we have observed primarily the rotation of entire membrane fragments, rather than the rotation of monomers/dimers, consistent with a highly aggregated enzyme. However, the observation of a small decrease in enzyme rotation in the protein–protein cross-linked samples relative to the K^+ and Na^+ samples suggests there may be some rotational freedom of smaller aggregates within these fragments. On the other hand, since the rotational mobility of the enzyme in any of the cationic environment samples is less restricted than in the 2D crystalline enzyme, the aggregation of ouabain-inactivated enzyme is probably not as organized or extensive as in 2D crystals, even in the presence of Mg^{2+}. It is well accepted that Na,K-ATPase stabilized in the E_2 conformation by Mg^{2+} and either vanadate or phosphate readily aggregates into 2D crystalline arrays. Since bound ouabain (OSL) also stabilizes the enzyme in an E_2 or E_2-like conformation (Askari et al., 1988; Forbush, 1983; Hansen, 1983, 1984), our ST-EPR results suggest strongly that the ouabain-inactivated Na,K-ATPase is extensively aggregated in the membrane. The role that Mg^{2+} plays in enhancing the ouabain-induced inhibition of Na,K-ATPase mobility (relative to Na^+ and K^+) remains unclear. These environment-dependent rotational differences are not likely to be the result of ligand-induced conformational changes in the protein, since Na,K-ATPase with bound ouabain (hence, OSL) is resistant to ligand-induced conformational changes (Askari et al., 1988). While it is possible that Mg^{2+} ions serve to stabilize the E_2 character of the ouabain-complexed enzyme [similar to their role in the formation of membrane crystals (Skriver et al., 1981; Maunsbach et al., 1988)], it is equally possible that these ions simply serve as weak electrostatic links between the lipids and proteins in pairs or groups of membrane fragments. Further study would be required to distinguish between these possibilities. Additional future studies should focus on (a) thorough analysis of OSL-Na,K-ATPase samples by electron microscopy, including freeze–fracture preparations, to better clarify the organizational state of the ouabain-inactivated enzyme and (b) EPR analysis of oriented, stacked membranes to determine the extent and nature of orientation of the nitroxides on the OSL-Na,K-ATPase.

Functional Implications for Ouabain Inactivation of Na,K-ATPase. It is well accepted that the Na,K-ATPase is fully functional as an $\alpha\beta$ promoter (Jørgensen 1985), though there is compelling evidence that the monomeric protein exists as functional dimers, or even larger oligomer networks, in the membrane to promote optimal functioning (Scheiner-Bobis et al., 1987; Cavieres 1987; Hansen et al., 1979; Askari et al.,

1988). Therefore, it is possible that the aggregation we observe in the OSL-Na,K-ATPase samples is simply reflecting spin label bound to preexisting aggregated enzyme. However, there is no physical or kinetic data in the literature to support a proposal that the entire complement of $\alpha\beta$-enzyme units in a given membrane fragment is functionally aggregated. Further, given that Esmann et al. (1989, 1990) have obtained reliable rotational correlation times for the partially active Na,K-ATPase, which are significantly shorter than those obtained from the ouabain-inactivated enzyme, it is reasonable to infer that ouabain promotes aggregation of the enzyme. Previous studies of protein–protein interactions and aggregation of Ca-ATPase monomers in SR have indicated that enforced interactions between enzyme units decrease Ca-ATPase activity and that aggregation serves to stabilize the enzyme in conformations resistant to enzymatic turnover (Lewis & Thomas, 1986; Squier & Thomas, 1988; Squier et al., 1988; Birmachu & Thomas, 1991; Mahaney et al., 1991; Voss et al., 1991). It is tempting to speculate, therefore, that aggregation of the inactivated enzyme may enhance ouabain's inhibitory effects by stabilizing the Na,K-ATPase in the ouabain-induced E_2-like conformation responsible for enzyme inactivation.

Future Applications for the Ouabain Spin Label. Apart from K^+ ions, cardiac glycosides are the only agents which bind specifically and with high affinity to the extracellular face of the Na,K-ATPase. Therefore, the ouabain spin label first described by Solomonson and Barber (1984) should be a valuable tool for further study of the rotational dynamics of the enzyme and for locating and studying the K^+ binding sites on the extracellular face of the Na,K-ATPase by NMR (Mildvan & Engle, 1972). However, dissociation of the label and its susceptibility to temperature-induced reduction may limit the label's utility at temperatures above 10–20 °C. Perhaps a simple redesign of the spin label will overcome these detrimental properties [e.g., replacing the six-member, fully saturated, and flexible nitroxide ring with an unsaturated five-member, planar, and rigid nitroxide (Esmann et al., 1990) and/or incorporating a photolyzable moiety into the spin-label structure (Forbush, 1983)] to keep the label tethered to the enzyme at higher temperatures.

Acknowledgments

We thank Jaymee Girard for synthesizing the ouabain spin label, Jan Redick and Bonnie Sheppard (Central Electron Microscopy) for assistance with our EM studies, Roger Morris and Marvin Grubb for expert instrumentation and electronics help, and Dr. John Stewart and Dr. Howard Kutchai for many helpful discussions concerning this work. We thank Nancy Cooke of Rocco Further Processing, Inc., Timberville, VA, for making fresh sheep kidneys available to us and her generous assistance in the procurement of the kidneys. We thank Dr. David Thomas, University of Minnesota, for the kind gift of the TPX sample tube and holder and for advice concerning ST-EPR data analysis. We thank Dr. Thomas Squier, University of Kansas, for his expert help and advice in working through the practical details of the ST-EPR experiment.

Registry No. ATPase, 9000-83-3; ouabain, 630-60-4.

References

Askari, A., Kakar, S. S., & Huang, W. (1988) *J. Biol. Chem. 263*, 235–242.

Barnett, R. E. (1970) *Biochemistry 9*, 4644, 4648.

Baroin, A., Thomas, D. D., Osborne, R., & Devaux, P. F. (1977) *Biochem. Biophys. Res. Commun. 78*, 442–449.

Bentley, D. (1983) Ph.D. Dissertation, State University of New York at Stony Brook.

Bigelow, D. J., Squier, T. C., & Thomas, D. D. (1986) *Biochemistry 25*, 194–202.

Birmachu, W., & Thomas, D. D. (1990) *Biochemistry 29*, 3904–3914.

Brotherus, J. R., Jacobsen, L., & Jørgensen, P. L. (1983) *Biochim. Biophys. Acta 731*, 290–303.

Cavieres, J. D. (1987) *FEBS Lett. 225*, 145–150.

Esmann, M. (1987) Ph.D. Dissertation, University of Aarhus.

Esmann, M., & Skou, J. C. (1984) *Biochim. Biophys. Acta 787*, 71–80.

Esmann, M., Horvâth, L. I., & Marsh, D. (1987) *Biochemistry 26*, 8675–8683.

Esmann, M., Hankovszky, H. O., Hideg, K., & Marsh, D. (1989) *Biochim. Biophys. Acta 978*, 209–215.

Esmann, M., Hankovszky, H. O., Hideg, K., Pedersen, P. A., & Marsh, D. (1990) *Anal. Biochem. 189*, 274–282.

Forbush, B. (1983) *Curr. Top. Membr. Transp. 19*, 167–201.

Fortes, P. A. G. (1977) *Biochemistry 16*, 531–540.

Gantzer, M. L., Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 4083–4088.

Goodno, C. C. (1982) *Methods Enzymol. 85*, 116–123.

Grisham, C. M., & Hutton, W. (1978) *Biochem. Biophys. Res. Commun. 81*, 1406–1411.

Grisham, C. M., Gupta, R., Barnett, R., & Mildvan, A. (1974) *J. Biol. Chem. 249*, 6738–6744.

Hansen, O. (1983) *Acta Pharmacol. Toxicol. 52*, 3–19.

Hansen, O. (1984) *Pharmacol. Rev. 36*, 143–163.

Hansen, O., Jensen, J., Norby, J., & Ottolenghi, P. (1979) *Nature 280*, 410–412.

Hegyvary, C., & Jørgensen, P. L. (1981) *J. Biol. Chem. 256*, 6296–6303.

Jørgensen, P. L. (1974) *Methods Enzymol. 32*, 277–290.

Jørgensen, P. L. (1985) *Biochem. Soc. Symp.*, 56–79.

Jørgensen, P. L., Skriver, E., Hebert, H., & Maunsbach, A. B. (1982) *Ann. N.Y. Acad. Sci. 402*, 207–225.

Kirley, T. L., Lane, L. K., & Wallick, E. T. (1986) *J. Biol. Chem. 261*, 4525–4528.

Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 6979–6984.

Lee, J. A., & Fortes, P. A. G. (1986) *Biochemistry 25*, 8133–8141.

Lewis, S. M., & Thomas, D. D. (1986) *Biochemistry 25*, 4615–4621.

Lingrel, J. B., Orlowski, J., Shull, M. M., & Price, E. M. (1990) *Prog. Nucleic Acid Res. Mol. Biol. 38*, 37–89.

Lowry, O., Rosebrough, N., Farr, A,. & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.

MacKnight, A. D. C., & Leaf, A. (1977) *Physiol. Rev. 57*, 510–573.

Mahaney, J. E., & Thomas, D. D. (1991) *Biochemistry 30*, 7171–7180.

Manella, C. A. (1982) *J. Cell Biol. 94*, 680–687.

Maunsbach, A. B., Skriver, E., Soderholm, M., & Hebert, H. (1988) *Prog. Clin. Biol. Res. 268A*, 39–56.

Mildvan, A. S., & Engle, J. L. (1972) *Methods Enzymol. 26*, 654–682.

Morse, P. D. (1987) *Biophys. J. 51*, 440a.

O'Connor, S. E., & Grisham, C. M. (1979) *Biochemistry 18*, 2315–2323.

O'Connor, S. E., & Grisham, C. M. (1980) *FEBS Lett. 118*, 303–307.

Perussi, J. R., Tinto, M. H., Nascimento, O. R., & Tabak, M. (1988) *Anal. Biochem. 173*, 289–295.

Popp, C. A., & Hyde, J. S. (1981) *J. Magn. Reson. 43*, 249-258.
Price, E. M., & Lingrel, J. B. (1988) *Biochemistry 27*, 8400-8408.
Quintanilha, A. T., Thomas, D. D., & Swanson, M. (1982) *Biophys. J. 37*, 68-69.
Rauckman, E. J., Rosen, G. M., & Griffeth, L. K. (1984) in *Spin Labelling in Pharmacology* (Holtzman, J. L., Ed.) Chapter 5, pp 175-190, Academic Press, Orlando, FL.
Saffman, P. J., & Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111-3113.
Scheiner-Bobis, G., Zimmerman, M., Kirch, U., & Schoner, W. (1987) *Eur. J. Biochem. 165*, 653-656.
Schwarz, D., Purrwitz, J., & Ruckpaul, K. (1982) *Arch. Biochem. Biophys. 216*, 322-328.
Segur, J. B., & Oberstar, H. E. (1951) *Ind. Eng. Chem. 43*, 2117-2120.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature 316*, 691-695.
Shull, G. E., Lane, L. K., & Lingrel, J. B. (1986) *Nature 321*, 429-431.
Skriver, E., Maunsbach, A. B., & Jørgensen, P. L. (1981) *FEBS Lett. 131*, 219-222.
Slie, W. M., Donfor, A. R., Jr., & Litovitz, T. A. (1966) *J. Chem. Phys. 44*, 3712-3718.
Solomonson, L. P., & Barber, M. J. (1984) *Biochem. Biophys. Res. Commun. 124*, 210-216.
Squier, T. C., & Thomas, D. D. (1986a) *Biophys. J. 49*, 921-935.
Squier, T. C., & Thomas, D. D. (1986b) *Biophys. J. 49*, 937-942.
Squier, T. C., & Thomas, D. D. (1988) *J. Biol. Chem. 263*, 9171-9177.
Squier, T. C., & Thomas, D. D. (1989) *Biophys. J. 56*, 735-748.
Squier, T. C., Hughes, S. E., & Thomas, D. D. (1988) *J. Biol. Chem. 263*, 9162-9170.
Stewart, J. M. M., & Grisham, C. M. (1988) *Biochemistry 27*, 4840-4848.
Stewart, J. M. M., Jørgensen, P. L., & Grisham, C. M. (1989) *Biochemistry 28*, 4595-4601.
Swanson, M. S., Quintanilha, A. T., & Thomas, D. D. (1980) *J. Biol. Chem. 255*, 7494-7502.
Thomas, D. D., & Hidalgo, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5488-5492.
Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006.
Thomas, R. C. (1972) *Physiol. Rev. 52*, 563-594.
Voss, J. C., Birmachu, W., Hussey, D., & Thomas, D. D. (1991) *Biochemistry 30*, 7498-7506.

Effect of B-Ring Substituents on Absorption and Circular Dichroic Spectra of Colchicine Analogues†

Erica A. Pyles,‡ Richard P. Rava,*,§ and Susan Bane Hastie*,‡

Department of Chemistry, State University of New York, Binghamton, New York 13902-6000, and George R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received September 6, 1991; Revised Manuscript Received November 26, 1991

ABSTRACT: Near-ultraviolet absorption and circular dichroic spectra of several B-ring derivatives of colchicine have been obtained in a variety of solvents. The spectra of the molecules in solvent were analyzed and compared with spectra of the molecules bound to tubulin. Absorption spectra of deacetamidocolchicine, deacetylcolchicine, demecolcine, and *N*-methyldemecolcine [B-ring substituents = H, NH_2, $NHCH_3$, and $N(CH_3)_2$, respectively] were analyzed by multiple differentiation of the spectrum. It was found that an amine substituent at the C-7 position on the B-ring of the colchicinoid affected the higher energy transition of the near-ultraviolet spectra of the colchicinoid in the absence of tubulin in a manner consistent with a hyperconjugative alteration of this transition. The fourth derivatives of the absorption spectra of all four molecules bound to tubulin were similar to each other and to colchicine. As was true in the case of colchicine, the negative near-ultraviolet circular dichroic band of the aminocolchicinoids was relatively unaffected by solvent, but the molar ellipticity of the band was greatly reduced with tubulin binding. It is concluded that the binding site environments of the B-ring analogues of colchicine, as probed by absorption and circular dichroic spectroscopy, are equivalent.

Colchicine is a potent antimitotic agent that acts by binding strongly to the protein tubulin [for reviews, see Luduena (1979), Brossi et al. (1988), and Hamel (1990)]. Tubulin binding induces alterations in the absorption, fluorescence, and circular dichroic (CD)[1] spectra of colchicine that have not been precisely mimicked in the absence of the protein (Bhattacharyya & Wolff, 1974; Detrich et al., 1981). For example, colchicine binding to tubulin is accompanied by a dramatic increase in colchicine fluorescence when the tubulin–colchicine

† This work was supported by a grant from the National Science Foundation (DMB 90-05614) to S.B.H. R.P.R. acknowledges the support of the MIT Laser Biomedical Research Center (RR02594).

* To whom correspondence should be addressed.
‡ State University of New York, Binghamton.
§ MIT.

[1] Abbreviations: CD, circular dichroism; UV, ultraviolet; DAAC, deacetamidocolchicine; NH_2-DAAC, deacetylcolchicine; NHMe-DAAC, demecolcine; NMe_2-DAAC, *N*-methyldemecolcine; DMF, dimethylformamide; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMEG buffer, 0.1 M Pipes, 1.0 mM $MgSO_4$, 2.0 mM EGTA, and 0.1 mM GTP, pH 6.90.

0006-2960/92/0431-2034$03.00/0