Lev, B., Ward, H., Keusch, G. T., & Pereira, M. E. A. (1986) *Science (Washington, D.C.) 232*, 71–73.
Lis, H., & Sharon, N. (1985) *Annu. Rev. Biochem. 55*, 35–67.
Marchase, R. B., Koro, L. A., Kelly, C. M., & McClay, D. R. (1982) *Cell (Cambridge, Mass.) 28*, 813–820.
Neufeld, E. F., & Ashwell, G. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 252–257, Plenum, New York.
Rhodes, J., & Prestwich, C. J. (1966) *Gut 7*, 509–514.
Sahagian, G. G., Distler, J. J., & Jourdian, G. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4289–4293.
Sharon, J., Morrison, S. L., & Kabat, E. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1420–1424.
Slodki, M. E., Ward, R. M., Boundy, J. A., & Cadmus, M. C. (1972) *Fermentation Technology Today, Proceedings of the IV International Fermentation Symposium*, pp 597–601, Society of Fermentation Technology, Osaka, Japan.
Sly, W. S., & Fischer, D. H. (1982) *J. Cell. Biochem. 18*, 67–85.
Springer, W. R., Cooper, D. N. W., & Barondes, S. H. (1984) *Cell (Cambridge, Mass.) 39*, 557–564.
Stoolman, L. M., Tenforde, T. S., & Rosen, S. D. (1984) *J. Cell Biol. 99*, 1535–1540.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Villalta, F., & Kierzenbaum, F. (1983) *Biochim. Biophys. Acta 736*, 39–44.
Ward, H. D., Lev, B. I., Keusch, G. T., & Pereira, M. E. A. (1987) *UCLA Symp. Mol. Cell. Biol., New Ser. 42*, 521–530.
Wilson, M. E., & Pearson, R. D. (1985) *Clin. Res. 33*, 423A.

Saturation-Transfer Electron Spin Resonance Studies on the Mobility of Spin-Labeled Sodium and Potassium Ion Activated Adenosinetriphosphatase in Membranes from *Squalus acanthias*†

Mikael Esmann,‡ László I. Horváth,§,‖ and Derek Marsh*,§

Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-3400 Göttingen, Federal Republic of Germany, and Institute of Biophysics, University of Aarhus, DK-8000 Aarhus, Denmark

Received May 20, 1987; Revised Manuscript Received August 18, 1987

ABSTRACT: The sodium and potassium ion activated adenosinetriphosphatase [(Na^+,K^+)-ATPase] in membranous preparations from *Squalus acanthias* has been spin-labeled on sulfhydryl groups after prelabeling with *N*-ethylmaleimide. Saturation-transfer electron spin resonance spectroscopy has been used to study the rotational motions of the labeled protein on the microsecond time scale. Effective rotational correlation times deduced from the diagnostic line-height ratios in the second-harmonic, 90° out-of-phase (V_2') spectra are much larger than those deduced from the spectral integrals, indicating the presence of large-scale segmental motions, in addition to rotation of the protein as a whole. Experiments involving controlled cross-linking of the protein by glutaraldehyde, as well as measurements of the line broadening of the conventional electron spin resonance spectra, support this interpretation. Both the spectral integrals and diagnostic line-height ratios are found to increase irreversibly with time on incubation at temperatures greater than 20 °C, corresponding to a decrease in the segmental motion of the protein and probably also in the overall protein rotation. The native enzyme displays a marked nonlinearity in the Arrhenius temperature dependence of the activity at temperatures above 20 °C, and the activity decreases with a half-life of ca. 70 min on incubation at 37 °C (but not on incubation at low temperature), paralleling the time- and temperature-dependent changes in the saturation-transfer spectra of the labeled protein. Both of these observations suggest that the changes observed in the molecular dynamics could correspond to functional properties of the protein. The effective rotational correlation time of the membranous enzyme, deduced from the low-field and high-field spectral line-height ratios using calibrations from isotropically rotating spin-labeled hemoglobin, lies in the region of 50 μs, implying an upper limit of $\tau_{R_\parallel} = 25$ μs for the true rotational correlation time of the protein.

The sodium and potassium ion activated adenosinetriphosphatase [(Na^+,K^+)-ATPase][1] (EC 3.6.1.8) is a membranous active-transport enzyme involved in maintaining ion levels and osmotic balance in the cell [for reviews, see, e.g., Skou (1965) and Glynn (1985)]. Functionally, the enzyme mediates the extrusion of three Na^+ ions and the uptake of two K^+ ions, coupled to the hydrolysis of one molecule of ATP. The catalytic transport cycle involves the formation of a phosphorylated enzyme intermediate and a conformational transition between the E_1 and E_2 forms of the enzyme, which display different affinities for Na^+ and K^+. Structurally, the

† Supported in part by the Danish Medical Research Council.
‡ University of Aarhus.
§ Max-Planck-Institut für biophysikalische Chemie.
‖ Permanent address: Institute of Biophysics, Biological Research Centre, H-6701 Szeged, Hungary.

[1] Abbreviations: (Na^+,K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.8); NEM, *N*-ethylmaleimide; MSH, 2-mercaptoethanol; GA, glutaraldehyde; EDTA, ethylenediaminetetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; SDS, sodium dodecyl sulfate; 5-MSL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy; ESR, electron spin resonance; STESR, saturation-transfer ESR; V_1, first harmonic ESR absorption signal detected in-phase with respect to the field modulation; V_2', second harmonic ESR absorption signal detected 90° out-of-phase with respect to the field modulation; kDa, kilodalton(s).

0006-2960/87/0426-8675$01.50/0

protein is composed of two different subunits, α (M_r 112K) and β (M_r 35K), which are present in equimolar amounts, although there is some uncertainty regarding the overall state of oligomerization. Whereas there is agreement that the detergent-solubilized $\alpha_2\beta_2$ dimer is active, the situation regarding the activity and stability of the monomer in detergent is less clear (Brotherus et al., 1983; Esmann, 1984; Jorgensen & Andersen, 1986). In addition, there are also features of the ligand binding to the membranous enzyme which can best be interpreted in terms of an interaction between monomers (Ottolenghi & Jensen, 1983).

A possible approach to the investigation both of conformational changes and of the state of aggregation of the protein is to study the microsecond time-scale motions of spin-labeled groups attached to the protein, using saturation-transfer electron spin resonance (STESR) (Hyde & Thomas, 1980; Thomas, 1985). Functionally significant conformational changes may involve large-scale molecular motions on this time scale, and the overall rotation rate of the protein, which is sensitive to the state of oligomerization of the protein, also lies in this time domain [see, e.g., Cherry (1985) and Garland and Johnson (1985)].

Labeling of the sulfhydryl groups of the (Na^+,K^+)-ATPase from shark rectal gland has been studied previously by Esmann (1982a,b). The nonessential SH groups can be modified selectively with *N*-ethylmaleimide in the presence of glycerol. This yields an enzyme in which the buried SH groups can be labeled selectively with the maleimide spin-label derivative. There is some heterogeneity in mobility of the labeled groups, but the preparation is suitable for study by saturation-transfer ESR, when combined with the STESR integral method (Horváth & Marsh, 1983). Unfortunately, the necessary labeling cannot be achieved without loss of overall ATPase activity, but at least phosphorylation activity to the E_1–P form of the enzyme is retained (Esmann & Klodos, 1983). The present work is concerned with the study of the segmental and overall rotational motion of the membranous (Na^+,K^+)-ATPase, with a consideration of the state of aggregation of the protein, and with correlation of changes in the microsecond rotational dynamics of the labeled protein with changes in activity of the native enzyme.

Materials and Methods

Materials. $C_{12}E_8$ was obtained from Nikko Chemicals, Tokyo, Japan. Glutaraldehyde (grade I) and *N*-ethylmaleimide were from Sigma, St. Louis, MO. Spin-labeled maleimide (5-MSL) was obtained from Syva, Palo Alto, CA.

Preparation of Enzyme. (Na^+,K^+)-ATPase-rich membranes were prepared from the rectal gland of *Squalus acanthias* according to the method of Skou and Esmann (1979). The membranes correspond to the "white pellet" enzyme in Table I of the latter reference. The specific (Na^+,K^+)-ATPase activity was typically 1100 μmol mg^{-1} h^{-1}, tested at 37 °C. Enzyme activity and protein determination were measured as described in Skou and Esmann (1979), and the membranous enzyme was stored in 20 mM histidine and 25% glycerol (pH 7.0). The purity of the enzyme with respect to the α and β subunits is about 50% estimated from the phosphorylation level and from SDS gel electrophoresis [see Skou and Esmann (1979)].

Prelabeling with N-Ethylmaleimide. The membrane-bound enzyme was prelabeled with *N*-ethylmaleimide (NEM) in the presence of K^+ and glycerol as previously described (Esmann, 1982a). Briefly, enzyme at 1 mg of protein/mL was incubated at 23 °C with 0.1 mM NEM in the presence of 150 mM KCl, 35% glycerol, and 5 mM CDTA for 60 min in 30 mM histidine (pH 7.0 at 23 °C). This procedure leads to incorporation of NEM in the α subunit as well as in the impurities of the membrane, but there is no loss of enzyme activity [see Esmann (1982b)]. The reason for the prelabeling procedure is to reduce the number of reactive SH groups and to block SH groups in the protein impurities of the preparation so that they cannot react with spin-label in a subsequent step. After prelabeling, the enzyme is washed extensively in 20 mM histidine and 25% glycerol (pH 7.0) and stored at −20 °C.

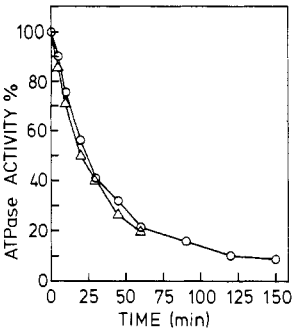

FIGURE 1: Time course of inactivation of the (Na^+,K^+)-ATPase activity by 5-MSL. (Na^+,K^+)-ATPase was incubated at 37 °C (△) or 20 °C (○) in the presence of 150 mM KCl, 5 mM CDTA, 3 mM ATP, and 30 mM histidine (pH 7.4 at 37 °C) with either 90 μM 5-MSL [(△) 37 °C] or 10 mM 5-MSL [(○) 20 °C]. At the time points indicated, an aliquot was withdrawn and mixed with a 4-fold excess (compared to 5-MSL) of 2-mercaptoethanol. The residual (Na^+,K^+)-ATPase activity is given as a percentage of the activity at time zero.

Spin-Labeling of the Enzyme. The prelabeled enzyme was reacted with maleimide spin-label (5-MSL) as previously described (Esmann, 1982a). Incubation with spin-label took place in the presence of 150 mM K^+ + 3 mM ATP at 37 or 23 °C (see Figure 1). At 37 °C, 80 μM 5-MSL leads to almost complete inactivation of the (Na^+,K^+)-ATPase activity within 30 min, whereas the inactivation rate is much lower at 23 °C (see Figure 1). A higher concentration of maleimide spin-label (ca. 1 mM) was therefore employed when the enzyme was spin-labeled at 23 °C. In both cases, the (Na^+,K^+)-ATPase activity is lost, but a partial activity—the ability to phosphorylate from Na^+ + ATP—is retained to a large extent [see Esmann and Klodos (1983)]. Control experiments have shown that all the spin-label is incorporated into the α subunit and, deduced from similar experiments with radioactively labeled NEM, the incorporation is 2–3 mol of spin-label per mole of α subunit. The reaction with 5-MSL was stopped by addition of a 2–10-fold excess of 2-mercaptoethanol (MSH), and the membranes were washed extensively, usually 3–4 times, in 20 mM histidine and 25% glycerol (pH 7.0) to remove unbound spin-label. A control experiment, where the spin-label had been prereacted with MSH, showed that no spin-label was present in the resulting membrane suspension after washing.

Cross-Linking with Glutaraldehyde. Glutaraldehyde cross-linking of the spin-labeled enzyme was performed as follows. The membranes were treated with 0.1–2% glutaraldehyde (GA) at 23 °C for 20 min, in the presence of 150 mM NaCl, 10 mM imidazole, and 1 mM EDTA (pH 7.5). The reaction with glutaraldehyde was quenched by addition of a large volume of 150 mM histidine, and the membranes were freed from residual glutaraldehyde by repeated centrifugations. The glutaraldehyde cross-linking of the subunits was complete with 0.5% GA, in the sense that no protein entered 5% SDS gels (see Figure 2). Furthermore, chromatography of the glutaraldehyde-treated membranes in SDS on Sepharose 2B (for which the exclusion limit is 4×10^7 D)

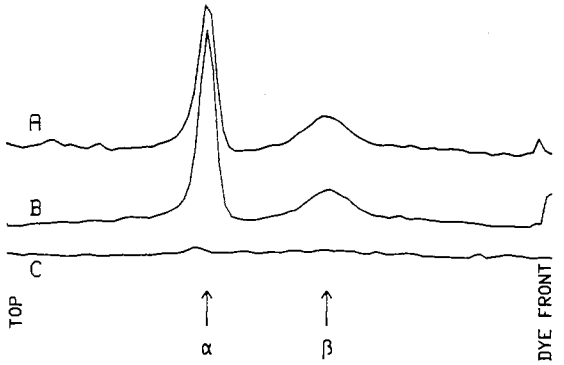

FIGURE 2: Scans of SDS gels of glutaraldehyde-treated enzyme and controls. The gels were stained with Coomassie Blue and scanned at 500 nm. Five micrograms of protein was applied to each gel. The top and dye front on the gels are indicated, together with the position of the α and β subunits. The gels are (A) control enzyme, (B) enzyme which has gone through the same manipulations as the cross-linked enzyme, but in the absence of glutaraldehyde, and (C) glutaraldehyde cross-linked enzyme (0.5% GA for 20 min).

revealed that the cross-linked protein, unlike the controls, eluted with the void volume.

$C_{12}E_8$ Solubilization. $C_{12}E_8$-solubilized membranes were prepared by addition of a concentrated solution of $C_{12}E_8$ to a pelleted membrane suspension, so that the $C_{12}E_8$/protein ratio was 4. No attempt to remove nonsolubilized material was made, but control experiments show that the (Na^+,K^+)-ATPase is fully solubilized at this detergent/protein ratio [see Esmann and Skou (1984)].

Polyacrylamide Gel Electrophoresis in SDS. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Weber and Osborn (1969). The protein was stained with Coomassie Blue using the method of Fairbanks et al. (1975), and gels were scanned at 500 nm in a Varian Cary Model 219.

Gel Filtration on Sepharose 2B in SDS. Control and cross-linked enzymes were subjected to gel filtration on a 30 × 1.5 cm column of Sepharose 2B (Pharmacia, Uppsala, Sweden) equilibrated in 20 mM sodium phosphate (pH 7.5) and 0.2% SDS. The membranes were solubilized in 2 g of SDS/g of protein, and the elution of the solubilized subunits was monitored at a wavelength of 280 nm. Control enzyme eluted at the total volume and the cross-linked enzyme at the dead volume.

ESR Measurements. For ESR measurement, membranes were suspended in the appropriate buffer, usually 100 mM NaCl, 1 mM CDTA, and 20 mM histidine (pH 7.4 at 37 °C), and the membranes were pelleted in this buffer. The pellets were freed from excess buffer, and about 10 μL of buffer was then added to the pellet. By gentle resuspension, the concentrated protein solution was transferred to a 1 mm i.d., 100-μL glass capillary, and the volume was trimmed to about 10 μL, yielding a sample height of 5 mm. This standardized sample geometry was used in all STESR experiments (Fajer & Marsh, 1982; Hemminga et al., 1984).

ESR spectra were recorded on a Varian E-12 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation. Samples were contained in the 1-mm-diameter 100-μL sealed glass capillaries within standard 4-mm-diameter quartz tubes containing light silicone oil for thermal stability. Conventional, in-phase, absorption ESR spectra (V_1 display) were recorded with a modulation frequency of 100 kHz and a modulation amplitude of 1.6 G p–p, at the same microwave power as used for the STESR measurements. STESR spectra were recorded in the second-harmonic, 90° out-of-phase, absorption mode (V_2' display) with a modulation frequency of

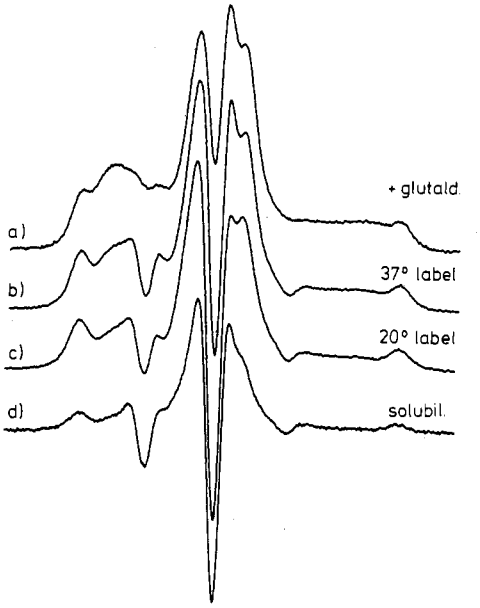

FIGURE 3: Second-harmonic, 90° out-of-phase STESR spectra (V_2') of 5-MSL spin-labeled (Na^+,K^+)-ATPase membranes at 0 °C. (a) Membranes labeled at 37 °C and cross-linked with 0.5% glutaraldehyde for 20 min. (b) Membranes labeled at 37 °C. (c) Membranes labeled at 20 °C. (d) Membranes labeled at 20 °C and solubilized in $C_{12}E_8$. Total scan width = 100 G.

50 kHz and a modulation amplitude of 5 G p–p. The microwave power was set for each sample and at each temperature so as to give an average H_1 microwave field over the sample of 0.25 G, according to the standardized protocol given in Fajer and Marsh (1982) and Hemminga et al. (1984). Integrals of the STESR spectra, normalized with respect to the conventional, in-phase spectral intensity, were evaluated as described in Horváth and Marsh (1983). Calibrations of the diagnostic STESR line-height ratios and normalized integral intensities, in terms of the rotational correlation times of spin-labeled hemoglobin, are given for our spectrometer system also in this latter reference. The rotational correlation times for these calibrations were calculated from the Debye equation for isotropic rotation diffusion:

$$\tau_R = 4\pi\eta r^3/3kT \quad (1)$$

where η is the viscosity of the solution and r = 29 Å is the molecular radius of hemoglobin. Spectral digitization and processing were performed using a PDP 11/10 dedicated computer and a Digital Equipment Corp. LPS system with VT-11 display. Further details on the ESR spin-label techniques are given in Marsh (1982).

RESULTS

Saturation-Transfer ESR (V_2'). Typical 90° out-of-phase second-harmonic absorption STESR spectra at 0 °C of the malemide-labeled (Na^+,K^+)-ATPase membranes in various states, and under different labeling conditions, are given in Figure 3. A progressive increase in rotational mobility is seen on going from the glutaraldehyde-fixed preparation, via membranes labeled at 37 and 20 °C, to membranes solubilized in $C_{12}E_8$ (Figure 3a–d, respectively). This is seen from the gradual decrease in relative intensity in the diagnostic spectral regions midway between the high-field and low-field turning points and the center of the spectra, respectively (Thomas et al., 1976). The temperature dependence of the STESR spectra from membranes labeled at 20 °C is given in Figure 4. The spectra indicate an increasing degree of rotational mobility with increasing temperature, as expected. However, an irre-

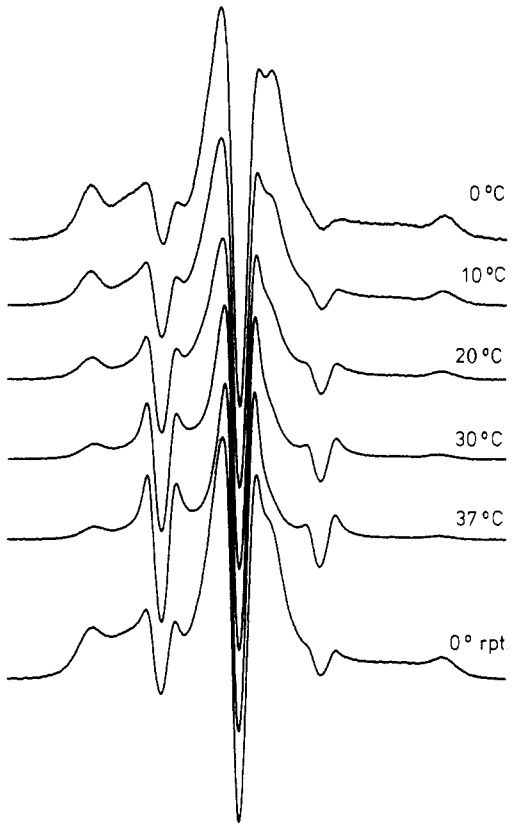

FIGURE 4: Temperature dependence of the second-harmonic, 90° out-of-phase (V_2') STESR spectra of (Na^+,K^+)-ATPase membranes labeled with 5-MSL at 20 °C. The bottom spectrum was recorded at 0 °C after the spectra from 0 to 37 °C were recorded. Total scan width = 100 G.

versible decrease in mobility is observed on returning to 0 °C after cycling to 37 °C (compare the upper and lower spectra in Figure 4). Prior to measurement, the sample had been prelabeled for 1 h at 23 °C and then spin-labeled for 3 h at 20 °C, but was otherwise kept at low temperatures and had never experienced higher temperatures. This temperature-dependent irreversibility accounts for the difference in mobility between the samples labeled at 37 °C and at 20 °C in Figure 3.

The STESR spectra in Figure 4 also reveal the presence of a mobile spin-label component (indicated by the sharp second-derivative-like peaks), which could not be eliminated entirely by NEM prelabeling. Because of its steeper temperature dependence, this second component becomes more obvious at the higher temperatures and considerably complicates the analysis of the saturation-transfer spectra. Overlap in the central region of the spectra means that the C′/C STESR diagnostic line-height ratio cannot be used, and the greater spectral overlap at the higher temperatures can even give rise to uncertainties in the low-field (L″/L) and high-field (H″/H) diagnostic regions. For this reason, the STESR spectra have also been analyzed by the integral method (Horváth & Marsh, 1983) with which it is possible to correct for the presence of the second component.

The temperature dependences of the normalized integrals, as defined by Horváth and Marsh (1983), and of the diagnostic line-height ratios, L″/L and H″/H as defined by Thomas et al. (1976), are given for normal, solubilized, and glutaraldehyde cross-linked membranes in Figure 5. In spite of the cross-linking, the glutaraldehyde-fixed samples display gradually decreasing normalized integrals and line-height ratios, corresponding to increasing rotational mobility with increasing temperature. The normal membranes display first decreasing,

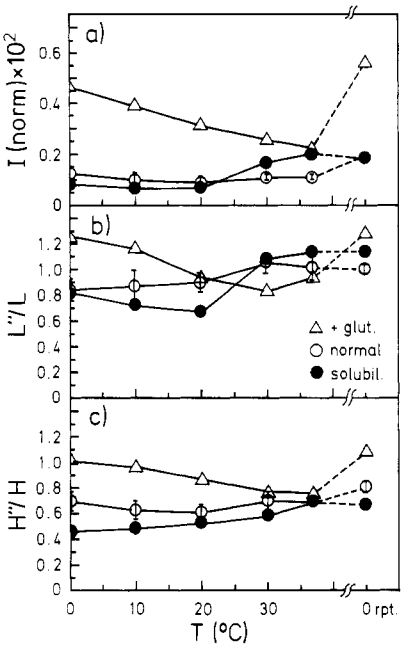

FIGURE 5: Temperature dependence of (a) the normalized integral, (b) the low-field line-height ratio, L″/L, and (c) the high-field line-height ratio, H″/H, in the V_2' STESR spectra of (Na^+,K^+)-ATPase membranes labeled with the 5-MSL spin-label. (△) Labeled at 37 °C and cross-linked with glutaraldehyde; (○) labeled at 23 °C; (●) labeled at 23 °C and solubilized in $C_{12}E_8$. Vertical bars represent standard deviation.

Table I: Temperature Dependence of the Normalized Integrals of the STESR Spectra of Maleimide Spin-Labeled (Na^+,K^+)-ATPase Membranes as a Function of Ionic Composition of the Suspending Medium[a]

	integral × 10^2					
salt	0 °C	10 °C	20 °C	30 °C	37 °C	0 °C rpt[b]
0.15 M NaCl	0.205	0.151	0.111	0.100	0.145	0.343
0.15 M KCl	0.210	0.153	0.123	0.102	0.177	0.345
5 mM $MgCl_2$	0.225	0.167	0.127	0.124	0.193	0.332
1 mM CDTA	0.215	0.153	0.118	0.093	0.089	0.288

[a] All buffer systems contained 30 mM histidine, pH 7.0. Membranes were labeled with 5-MSL at 37 °C for 60 min. [b] 0 °C rpt: repeat measurement at 0 °C after measurements at 0–37 °C with increasing temperature.

or approximately constant, normalized integrals and line-height ratios up to 20 °C, which then increase with further increase in temperature. The solubilized membranes display a similar temperature dependence, except that the initial decrease is less and the subsequent increase is greater than observed with the untreated membranes. All three samples display an irreversible increase in normalized integrals and line-height ratios on returning to 0 °C after cycling to 37 °C (see 0 rpt in Figure 5).

Cross-linking experiments involving different glutaraldehyde concentrations and various times of incubation revealed a progressive change in the STESR parameters, indicating a decreasing rotational mobility with increasing GA concentration and length of incubation (data not shown). A rapid decrease in enzymatic activity was observed on incubation with GA, which preceded, to a considerable extent, the changes in the STESR spectra. This suggests that the former might result from intramolecular cross-linking and the latter might result preferentially from intermolecular cross-linking, i.e., from a slowing down of the rate of overall protein rotation.

The dependence of the temperature-induced increase in STESR integrals on the ionic composition of the suspending buffer was also investigated. The temperature dependence of the normalized STESR integrals of 37 °C labeled membranes

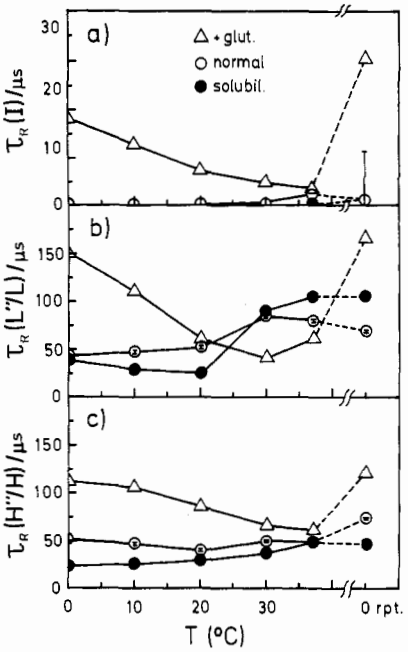

FIGURE 6: Effective rotational correlation times, τ_R, of spin-labeled (Na^+,K^+)-ATPase deduced from the STESR measurements of Figure 5. (a) From the normalized integral, (b) from the low-field line-height ratio, and (c) from the high-field line-height ratio. (△) Membranes labeled at 37 °C and cross-linked with glutaraldehyde; (○) membranes labeled at 23 °C; (●) membranes labeled at 23 °C and solubilized with $C_{12}E_8$.

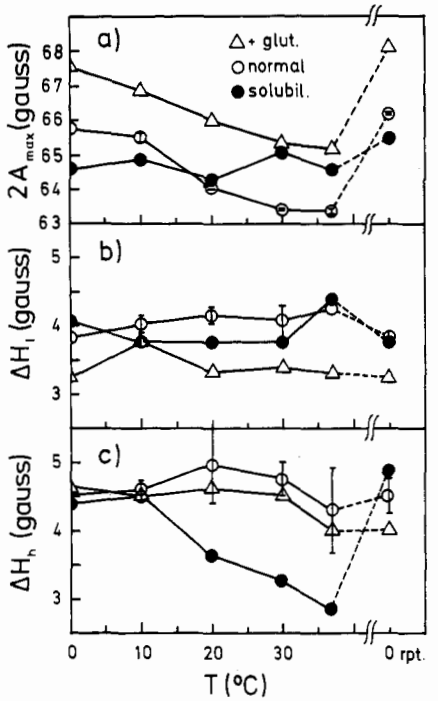

FIGURE 7: Temperature dependence of (a) the outer hyperfine splitting, $2A_{max}$, (b) the low-field line width, ΔH_l, and (c) the high-field line width, ΔH_h, in the conventional ESR spectra of (Na^+,K^+)-ATPase membranes labeled with the 5-MSL spin-label. (△) Labeled at 37 °C and cross-linked with glutaraldehyde; (○) labeled at 23 °C; (●) labeled at 23 °C and solubilized in $C_{12}E_8$. Vertical bars represent standard deviation.

is given in Table I. Very similar values are obtained at all temperatures for membranes suspended in 0.15 M NaCl or KCl, or in 5 mM $MgCl_2$. The integrals decrease to a minimum at 20–30 °C and then increase on further heating to 37 °C. An irreversible increase in integral is then observed on returning to 0 °C, which is of approximately the same size for all three cases. In the absence of added salts (1 mM CDTA), the integrals decrease monotonically with increasing temperature, although to a much smaller extent at the higher temperatures. On returning to 0 °C, an irreversible increase in the integral is again observed, but to a considerably reduced extent compared to the samples with added salt.

The temperature dependence of the effective correlation times deduced from the normalized integrals and diagnostic line-height ratios, using calibrations from the isotropic rotation of spin-labeled hemoglobin in glycerol–water solutions (Horvâth & Marsh, 1983), is given in Figure 6. In general, the dependences follow those of the STESR parameters in Figure 5. However, important distinctions are seen between the absolute values of the effective correlation times deduced from the different parameters. Although the values obtained from the two line-height ratios are rather similar, the effective correlation times from the normalized integrals are much lower. In the case of the normal and solubilized membranes, the latter are even below the minimum of the range of correlation times which may be determined from the calibrations. The differences can probably be attributed to segmental motion of the label relative to the protein, as discussed later, although overlap of the mobile component in the diagnostic regions of the line-height ratios may also play a minor part.

Conventional ESR. The possibility of segmental motion has been investigated by using the conventional ESR spectra of the labels in the various membrane preparations. The temperature dependence of the hyperfine splitting, $2A_{max}$, and line widths at half-height, ΔH_l and ΔH_h, of the outer extrema of the slow-motion component is given in Figure 7. In general, the temperature dependence reflects that of the STESR parameters in Figure 5, taking into account the fact that increasing line widths and decreasing line splittings reflect increasing rotational mobility in the slow-motional regime of conventional ESR. Calibrations of these parameters in terms of simulations of the conventional ESR spectra for slow isotropic motion have been given by Freed (1976). The temperature dependence of the resulting effective rotational correlation times is given in Figure 8. The values deduced from all three parameters are similar and indicate that the maleimide spin probe undergoes some segmental motion with effective correlation times in the range of tens of nanoseconds. The extent of this limited segmental motion is, however, not clear from the slow-motion analysis.

Enzyme Activity. Corresponding experiments have been carried out on the protein function, to compare with the results on the protein rotational motion. An Arrhenius plot of the temperature dependence of the (Na^+,K^+)-ATPase activity of the native enzyme, at saturating ATP concentrations, is given in Figure 9. It is seen that the plot is markedly nonlinear, with departures from linearity first occurring at temperatures greater than 20 °C. The changes are such that a lower effective activation energy is found at the higher temperatures. The effects of incubation at 0 and at 37 °C on the native enzyme activity are given in Table II. Enzyme stored in 25% glycerol at low temperature was diluted into standard buffer and then incubated at 0 or at 37 °C. As can be seen from Table II, whereas there is little loss of activity at 0 °C, the activity decreases with a half-time of 70 min on incubation at 37 °C, to a limiting level which is approximately 50% of the original value. The kinetics of this inactivation are closely similar to those of the changes observed in the STESR spectra of the labeled enzyme at higher temperatures.

DISCUSSION

Motional Heterogeneity and the STESR Integral. The

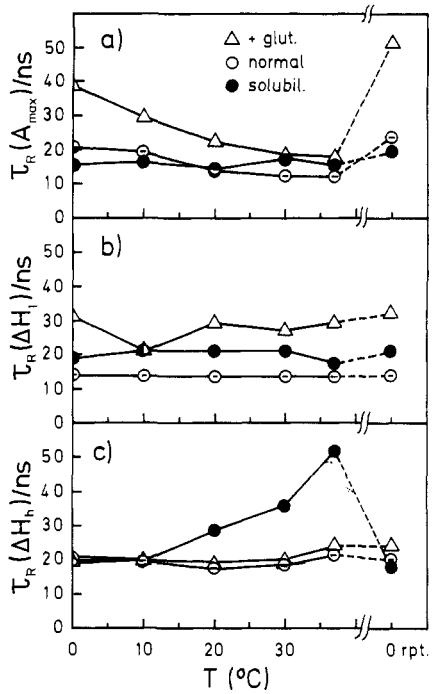

FIGURE 8: Effective rotational correlation times of the 5-MSL label covalently linked to the (Na^+,K^+)-ATPase, deduced from the conventional ESR data of Figure 7. (a) From the outer hyperfine splitting, (b) from the low-field line width, and (c) from the high-field line width. (△) Membranes labeled at 37 °C and cross-linked with glutaraldehyde; (○) membranes labeled at 23 °C; (●) membranes labeled at 23 °C and solubilized in $C_{12}E_8$.

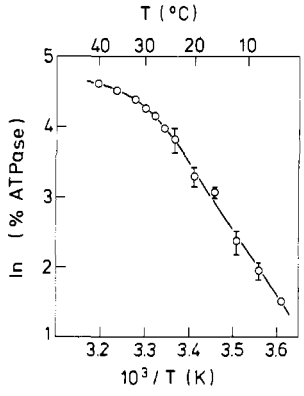

FIGURE 9: Arrhenius plot of the temperature dependence of the ATP hydrolyzing activity of the native (Na^+,K^+)-ATPase in membranes from *Squalus acanthias*. (Na^+,K^+)-ATPase activity was measured at 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, and 3 mM ATP in 30 mM histidine (pH 6.8–7.4). The pH optimum for the ATP hydrolysis decreases with decreasing temperature from about pH 7.4 at 37 °C to about pH 6.8 at 4 °C. Specific activities, at the pH optimum, are normalized to 100% at 40 °C. Vertical bars represent standard deviation.

spin-labeling of the –SH groups of (Na^+,K^+)-ATPase membranes from *Squalus acanthias* has been investigated in detail by Esmann (1982a,b). Under all labeling conditions, a mobile spin-label component is obtained in the conventional ESR spectra, in addition to the dominant immobilized spectral component with an outer hyperfine splitting of approximately 64–65 G. In the present work, labeling conditions were used which minimize the proportion of the mobile spectral component, but it was not possible to eliminate it completely. By use of the labeling protocol described under Materials and Methods, a preparation is obtained which gives rise to highly reproducible conventional ESR spectra. The labeled enzyme has only 5–10% of the initial (Na^+,K^+)-ATPase activity, but the ability to phosphorylate is retained up to 50–80% of the control values. [Maleimide spin-labeled preparations which retain (Na^+,K^+)-ATPase activity give rise to ESR spectra which are dominated by contributions from the very mobile groups and hence are unsuitable for saturation-transfer spectroscopy (Esmann, 1982a).]

The presence of the mobile spectral component complicates interpretation of the STESR spectra because of overlap in the diagnostic regions for determination of line-height ratios, and necessitates use of the STESR integral method (Horvâth & Marsh, 1983). The lack of correspondence between the effective rotational correlation times obtained from the integral and line-height ratio analyses may possibly be attributed to more rapid segmental motion of the label relative to the protein backbone. Such a motion could reduce the overall STESR intensity, while retaining at least part of the STESR spectral line shape characteristic of the slow rotational modes of the protein as a whole. Since the STESR integral is a relatively new method of spectral analysis, it has been used little to date, and thus, it is not clear to what extent such effects of segmental motion may appear in other systems investigated by STESR spectroscopy. For the Ca^{2+}-ATPase, one of the few systems so far investigated by the integral method, the effective correlation time derived from the STESR integral is considerably reduced relative to that derived from the line-height ratios, although not to as great an extent as found here for the (Na^+,K^+)-ATPase (Lewis & Thomas, 1986).

Segmental Motion. Supportive evidence for the existence of segmental motion comes from the experiments involving controlled cross-linking of the protein with glutaraldehyde. Practically complete cross-linking does not entirely abolish all rotational motion on the microsecond time scale (see Figure 6), although the considerable increase in effective correlation time relative to the untreated membranes clearly demonstrates that the STESR spectra are sensitive to overall protein rotation. The presence of a very marked temperature dependence of the effective correlation time in the glutaraldehyde-fixed membranes also strongly suggests the presence of independent segmental motion of the labeled groups. It is possible that segmental motion is similarly present in the maleimide spin-labeled Ca^{2+}-ATPase, since microsecond rotational motion was still detected in two-dimensional crystalline samples induced by decavanadate (Lewis & Thomas, 1986). Further evidence for segmental motion in the maleimide spin-labeled (Na^+,K^+)-ATPase comes from the analysis of the conventional ESR spectra. The results of Figures 7 and 8 indicate that the spin-labeled groups do in fact have some residual rotational mobility in the slow-motional regime of conventional ESR spectroscopy ($\tau_R \lesssim 10^{-7}$ s). None of the systems studied gives rise to a conventional rigid-limit ESR spectrum.

Time- and Temperature-Dependent Changes in Mobility. A particularly noteworthy feature of the results is the lack of reversibility after cycling to 37 °C. For normal membranes, this effect becomes obvious at temperatures above 20 °C (see Figure 5 and Table I) which led us to investigate membranes labeled both at 20 °C and at 37 °C. The STESR parameters of the 37 °C labeled membranes invariably indicated less mobility than those of membranes labeled at 20 °C (cf. Figure 3), and prolonged incubation of 20 °C labeled membranes at 37 °C gave rise to a progressive decrease in mobility. Since the effect was also evident in cross-linked membranes (see Figure 6), it must correspond at least in part to a decrease in mobility of internal motional modes within the protein (cf. also Figure 8). It is interesting to note that prolonged incubation at 37 °C gives rise to a progressive decrease in ATPase activity

Table II: Activity of Native (Na^+,K^+)-ATPase as a Function of Time of Incubation at 0 and 37 °C[a]

time (h)	activity (%) 0 °C	37 °C
0	100	100
0.5	90	79
1	88	72
2	89	59
3	88	56
4	85	53

[a] (Na^+,K^+)-ATPase membranes were incubated at 0 or 37 °C in 100 mM NaCl, 1 mM CDTA, and 30 mM histidine (pH 7.4) for the indicated times, and the specific (Na^+,K^+)-ATPase activity was subsequently assayed at 37 °C.

on a time scale similar to that of the changes in the STESR spectra (cf. Table II) and perhaps more significantly that a change in slope of the Arrhenius plot of the enzyme activity occurs in the range of 25–30 °C (see Figure 9). These latter results suggest that the internal mobility observed by STESR may be correlated with enzymatic function. It should be noted that the temperature irreversibility in the STESR parameters does not correspond to a reduction in the contribution from mobile groups as suggested for the Ca^{2+}-ATPase (Bigelow et al., 1986; King & Quinn, 1983). Although some spin-label reduction does occur at higher temperatures with the (Na^+,K^+)-ATPase membranes, the extent is relatively small and does not give rise to an appreciable change in the relative proportion of the mobile component. On the other hand, it cannot be excluded that changes similar to those observed here also occur in the Ca^{2+}-ATPase during the incubation protocol used by Bigelow et al. (1986) to eliminate time-dependent effects in the STESR spectra of maleimide spin-labeled preparations.

The time- and temperature-dependent changes in the STESR spectra are even greater in the detergent-solubilized preparations than in the membranous preparations (cf. Figures 5 and 6). This suggests that protein aggregation may also be involved in the time-dependent process, as well as the decrease in internal mobility, since the protein is known to aggregate in detergent solution (Esmann, 1984). The high protein concentrations required for STESR are almost certain to accelerate this aggregation process.

Rotational Diffusion and Oligomer State. Information regarding the state of aggregation of the protein in the membrane can, in principle, be determined from the rotational diffusion coefficient, $D_{R_\parallel}$. An expression for the rotational diffusion coefficient of a cylindrical protein, predicted from hydrodynamic theory, has been given by Saffman and Delbrueck (1975), and the extension to proteins of elliptical cross section has been given by Jähnig (1986):

$$D_{R_\parallel} = \frac{kT}{4\pi\eta abh} \frac{2a/b}{1 + (a/b)^2} \qquad (2)$$

where a and b are the semi-axes of the cylinder ($a > b$), h is the height of the membrane-spanning region of the cylinder, η is the effective viscosity within the membrane, k is Boltzmann's constant, and T is the absolute temperature. A rotational correlation time corresponding to this rotational diffusion coefficient can be defined by (Robinson & Dalton, 1980)

$$\tau_{R_\parallel} = 1/6D_{R_\parallel} \qquad (3)$$

Values appropriate to the (Na^+,K^+)-ATPase monomer can be calculated from the cross-sectional dimensions obtained by electron microscopy and image reconstruction (Hebert et al., 1985), together with a value of $h = 45$ Å. For values of the effective membrane viscosity within the realistic range η = 2.4–5 P [see, e.g., Cherry and Godfrey (1981)], the calculated rotational correlation time for the monomer varies from 5 to 10 μs.

As already discussed, the values of the effective rotational correlation time obtained from the STESR integrals cannot be used for comparison, because they are unduly affected by segmental motion. Values of the effective rotational correlation time deduced from the line-height ratios are invariably greater (see Figure 6) and more likely to reflect the rotational motion of the protein as a whole. In any case, it is unlikely that the effective rotational correlation times will be increased by segmental motion; therefore, the values given in Figure 6b,c are likely to be lower estimates. Robinson and Dalton (1980) have performed theoretical line-shape simulations appropriate to anisotropic rotational diffusion for first-harmonic dispersion STESR spectra and have related the effective rotational correlation time, τ_R^{eff}, deduced from isotropic model systems to the true value of $\tau_{R_\parallel}$. For highly anisotropic rotation ($D_{R_\parallel} \gg D_{R_\perp}$) in the correlation time range relevant here, it was found that

$$\tau_R^{eff} = 1/\{3[D_{R_\parallel} \sin^2\theta + D_{R_\perp}(1 + \cos^2\theta)]\} \qquad (4)$$

where $D_{R_\perp}$ is the element of the diffusion tensor corresponding to rotations about an axis perpendicular to the principal axis of rotation and θ is the angle between the nitroxide z axis and the principal rotation axis. Thus, for $\theta = 90°$, the effective correlation time is $\tau_R^{eff} = 2\tau_{R_\parallel}$ and increases with decreasing θ until for $\theta = 0°$ it becomes totally insensitive to rotations about the parallel axis [cf. Marsh (1980)]. This strong dependence on the spin-label orientation clearly introduces a considerable uncertainty in the analysis of the experimental data. The observed effective correlation times of the membrane-bound protein are in the region of 50 μs, which means that all spin-label groups would have to be oriented within 30° of the principal diffusion axis if it were definitely to be concluded that the protein was present as a monomer in the membrane. (Note that more than one spin-label is incorporated per subunit.) The results from the glutaraldehyde cross-linking experiments suggest that the nitroxide axes do not have an orientation very close to $\theta = 0°$, since the effective correlation times deduced from the low-field and high-field line-height ratios are substantially affected (whereas the low value deduced from the integral for the glutaraldehyde-treated membranes suggests that the segmental motion is relatively unaffected by cross-linking).

An upper limit for the rotational correlation time of the protein deduced from the experimental data is, according to eq 4, $\tau_{R_\parallel} = 25$ μs, which is considerably greater than the maximum value estimated for the protein monomer. This value would correspond to a $\theta = 90°$ orientation of the nitroxide axes. The rotational correlation time for the dimer, predicted from eq 2, lies in the range $\tau_{R_\parallel}$ = 16–34 μs, depending on the effective membrane viscosity. Radiation inactivation experiments on the membranous (Na^+,K^+)-ATPase have yielded a target size which was found to correspond to the M_r 264K dimer (Ottolenghi & Ellory, 1983). An effective correlation time of 60 μs has previously been obtained from STESR measurements on the Ca^{2+}-ATPase in sarcoplasmic reticulum (Thomas & Hidalgo, 1978) and a value of 50 μs for the same protein reconstituted in egg phosphatidylcholine at 30 °C (Andersen et al., 1981). In these latter cases, the protein dimer is expected to have a relative molecular mass

of 220 kDa. For further comparison of the (Na^+,K^+)-ATPase and the Ca^{2+}-ATPase, see Norby (1987).

Solubilized Enzyme. The viscosity of a 300 mg/mL solution of $C_{12}E_8$ in the standard histidine/NaCl/CDTA buffer has been measured to be 24 cP at 4 °C. The Stokes radius of the solubilized enzyme, measured previously by hydrodynamic methods, has been found to be 66.4 Å for the $\alpha\beta$ monomer and 77.4 Å for the $\alpha_2\beta_2$ dimer (Esmann et al., 1980). Using eq 1 for isotropic rotational diffusion would then predict correlation times of 8 and 12 μs for the solubilized monomer and dimer, respectively. The rotational correlation time deduced from the STESR line-height ratios in Figure 6 is approximately 25 μs. The high protein concentration used for the STESR measurements probably increases the viscosity considerably over that of the $C_{12}E_8$ solution alone. This would then give reasonable agreement between the predicted and measured correlation times, although it is not possible to decide whether the solubilized protein is in the monomeric or dimeric form under the conditions of the STESR experiment. Unfortunately, the amount of protein required to make a reliable measurement of the viscosity of the solutions used for the STESR measurements is prohibitively high.

Conformational State of the Enzyme. The reason for the low (Na^+,K^+)-ATPase activity of the labeled enzyme is probably that the equilibrium between the E_1–P and the E_2–P forms is displaced toward the E_1–P form, in the sense that the sensitivity to dephosphorylation by K^+ is greatly reduced (Esmann & Klodos, 1983). Using eosin as a probe of the conformational state of the labeled enzyme, it has also been shown that the nonphosphorylated enzyme undergoes the transition from the E_1 form to the E_2 form, but with a very reduced affinity for K^+ (M. Esmann, unpublished results). For example, an approximately 20-fold higher K^+ concentration is required to displace eosin from the labeled enzyme than from the control enzyme. It can, however, safely be assumed that the enzyme is in the E_2 form at 50–150 mM KCl and in the E_1 form in 50–150 mM NaCl. The latter conclusion has interesting implications for the results reported in Table I. In the experiments in which 0.15 M NaCl is replaced by 0.15 M KCl in the suspension medium, no difference is observed in the integrals of the STESR spectra, nor in the STESR line-height ratios. This suggests that the E_1 and the E_2 forms of the enzyme have the same size, i.e., have essentially the same oligomeric states. Similarly, the segmental motion detected by STESR is presumably also the same in the two conformational states of the labeled enzyme.

Summary. In summary, time- and temperature-dependent changes are seen in the saturation-transfer spectra of the maleimide-labeled (Na^+,K^+)-ATPase. These changes have a threshold at approximately 20–30 °C and correspond to changes in the protein segmental motion, and possibly also in the state of protein aggregation. Corresponding changes are observed in the activity of the native enzyme. Nonlinearities appear in the Arrhenius law temperature dependence at temperatures above this threshold value, and the activity decreases on incubation at 37 °C with a time dependence which is similar to that for the changes in the STESR spectra of the labeled enzyme. Uncertainties in the orientation of the spin-labels relative to the protein rotation axis preclude making positive statements regarding the aggregation state of the membranous enzyme.

ACKNOWLEDGMENTS

We thank Angilina Tepper for expert technical assistance.

Registry No. ATPase, 9000-83-3.

REFERENCES

Andersen, J. P., Fellmann, P., Moeller, J. P., & Devaux, P. F. (1981) *Biochemistry 20*, 4928–4936.

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

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

Cherry, R. J. (1985) in *Spectroscopy and the Dynamics of Molecular Biological Systems* (Bayley, P. M., & Dale, R. E., Eds.) pp 79–93, Academic Press, London.

Cherry, R. J., & Godfrey, R. E. (1981) *Biophys. J. 36*, 257–276.

Esmann, M. (1982a) *Biochim. Biophys. Acta 688*, 251–259.

Esmann, M. (1982b) *Biochim. Biophys. Acta 688*, 260–270.

Esmann, M. (1984) *Biochim. Biophys. Acta 787*, 81–89.

Esmann, M., & Klodos, I. (1983) *Curr. Top. Membr. Transp. 19*, 349–352.

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

Esmann, M., Christiansen, C., Karlsson, K.-A., Hansson, G. C., & Skou, J. C. (1980) *Biochim. Biophys. Acta 603*, 1–12.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2617–2624.

Fajer, P., & Marsh, D. (1982) *J. Magn. Reson. 49*, 212–224.

Freed, J. H. (1976) in *Spin Labeling. Theory and Applications* (Berliner, L. J., Ed.) Vol. 1, pp 53–132, Academic Press, New York.

Garland, P. B., & Johnson, P. (1985) in *Spectroscopy and the Dynamics of Molecular Biological Systems* (Bayley, P. M., & Dale, R. E., Eds.) pp 95–118, Academic Press, London.

Glynn, I. M. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) 2nd ed., Vol. 3, pp 35–114, Plenum Press, New York.

Hebert, H., Skriver, E., & Maunsbach, A. B. (1985) *FEBS Lett. 187*, 182–186.

Hemminga, M. A., de Jager, P. A., Marsh, D., & Fajer, P. (1984) *J. Magn. Reson. 59*, 160–163.

Horváth, L. I., & Marsh, D. (1983) *J. Magn. Reson. 54*, 363–373.

Hyde, J. S., & Thomas, D. D. (1980) *Annu. Rev. Phys. Chem. 31*, 293–317.

Jähnig, F. (1986) *Eur. J. Biophys. 14*, 63–64.

Jorgensen, P. L., & Andersen, J. P. (1986) *Biochemistry 25*, 2889–2897.

King, M. D., & Quinn, P. J. (1983) *J. Bioenerg. Biomembr. 15*, 135–150.

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

Marsh, D. (1980) *Biochemistry 19*, 1632–1637.

Marsh, D. (1982) in *Techniques in Lipid and Membrane Biochemistry* (Metcalfe, J. C., & Hesketh, T. R., Eds.) Vol. B4/II, pp B426/1–B426/44, Elsevier, Ireland.

Norby, J. G. (1987) *Chem. Scr.* (in press).

Ottolenghi, P., & Ellory, J. C. (1983) *J. Biol. Chem. 258*, 14895–14907.

Ottolenghi, P., & Jensen, J. (1983) *Biochim. Biophys. Acta 727*, 89–100.

Robinson, B. H., & Dalton, L. R. (1980) *J. Chem. Phys. 72*, 1312–1324.

Saffman, P. G., & Delbrueck, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113.

Skou, J. C. (1965) *Physiol. Rev. 3*, 596–617.
Skou, J. C., & Esmann, M. (1979) *Biochim. Biophys. Acta 567*, 436–444.
Thomas, D. D. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) 2nd ed., Vol. 1, pp 287–312, Plenum Press, New York.
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*, 3006–3024.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Gene Family of Male-Specific Testosterone 16α-Hydroxylase (C-P-450$_{16\alpha}$) in Mouse Liver: cDNA Sequences, Neonatal Imprinting, and Reversible Regulation by Androgen

Garry Wong, Kaname Kawajiri, and Masahiko Negishi*

Laboratory of Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, North Carolina 27709

Received June 10, 1987; Revised Manuscript Received July 23, 1987

ABSTRACT: The cDNA clone p16α-1 for the male-specific isozyme (C-P-450$_{16\alpha}$)[1] of testosterone 16α-hydroxylase in livers of 129/J mice [Harada, N., & Negishi, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2024–2028] and two additional full-length cDNAs overlapping with p16α-1 (p16α-2 and p16α-16) were sequenced. p16α-2 contained a single open reading frame of 1512 nucleotides, consisting of 71 base pairs of the 5′-noncoding region and 63 base pairs of the 3′-noncoding region with an additional poly(A) tract. From this DNA sequence, C-P-450$_{16\alpha}$ was deduced to contain 504 amino acids with a calculated molecular mass of 56 948 daltons. p16α-1 showed a nucleotide sequence identical with that of p16α-2 but lacked nine amino acid residues from the N-terminus. Another cDNA clone, p16α-16, also exhibited the same coding sequence with the exception of a 142 base pair deletion spanning from nucleotide 853 to nucleotide 994 of p16α-2. This deletion seems to be a whole exon of this gene, resulting in a shift of reading frame and an early termination codon at 10 amino acid residues from the deletion. The expected translation product of this mRNA is calculated to be 294 amino acids and 33 300 daltons. The putative poly(A) addition signal AATAAA is present for all three clones, but there are polymorphisms in the start sites of polyadenylation. Amino acid alignment with P-450b, P-450 M1, and P-450c revealed that P-450$_{16\alpha}$ is 39.8%, 40.7%, and 31.5% homologous, respectively. Five highly homologous genes were selected from a genomic DNA library of BALB/cJ mice by plaque hybridization to the cDNA and characterized. An oligonucleotide only specific for p16α-1 and its gene was prepared from p16α-2 and its gene. The p16α-1 and the specific oligonucleotide were hybridized with liver poly(A)+ RNA from control and testosterone propionate treated adult 129/J mice that had been castrated at day 1 of birth or at adult age. The results indicated that p16α-1 recognizes at least two mRNAs in terms of their regulation by androgen; one is reversibly regulated and the other is neonatally imprinted in expression in adult livers. The two differentially regulated C-P-450$_{16\alpha}$-dependent testosterone 16α-hydroxylase activities in the liver microsomes from these mice were also detected and associated with the hybridizable mRNA levels. A hybridization of the specific oligonucleotide probe with the poly(A)+ RNA concluded that p16α-1 and p16α-2 encode C-P-450$_{16\alpha}$, which is reversibly regulated.

As with other liver enzymes and proteins, such as drug oxidases, monoamine oxidase, prolactin receptor, and major mouse urinary protein (MUP), steroid hydroxylase activities in microsomes exhibit a marked sexual dimorphism in rodents (Colby, 1980; Roy & Chatterjiee, 1983). For instance, testosterone 16α-hydroxylase activity in mouse liver microsomes is known to be male predominant in some inbred strains, such as 129/J, whereas the activity is expressed at a high level in females as well as in males of other inbred strains, such as BALB/cJ and C57BL/6J (Ford et al., 1979; Harada & Negishi, 1984a; Harada & Negishi, 1984b; Noshiro et al., 1986). In inbred mice, such as BALB/cJ, apparent 16α-hydroxylase activity in microsomes is the sum of at least two sexually differentiated isozymes (forms of P-450); one (called C-P-450$_{16\alpha}$)[1] is predominantly expressed in male mice and the other (called I-P-450$_{16\alpha}$) is female specific (Harada & Negishi, 1984b; Devore et al., 1985; Noshiro et al., 1986). The strain differences in the activity among the inbred strains of mice

* Address correspondence to this author at the Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709.

[1] When the form of P-450 specific for testosterone 16α-hydroxylase activity was purified from 129/J male mice, we named this form P-450$_{16\alpha}$ (Harada & Negishi, 1984a). Subsequently, another P-450 was purified from phenobarbital-treated 129/J female mice, which is also specific for testosterone 16α-hydroxylase activity (Devore et al., 1985), and the immunochemical evidence suggested that this P-450 is constitutively female specific. Therefore, in order to distinguish the two isozymes, the P-450$_{16\alpha}$ was renamed C-P-450$_{16\alpha}$ (Noshiro et al., 1986a).