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Local translational diffusion rates of membranous Na^+,K^+-ATPase measured by saturation transfer ESR spectroscopy

(spin label/spin exchange/spin-lattice relaxation)

MIKAEL ESMANN* AND DEREK MARSH†‡

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

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ABSTRACT **Diffusion-controlled Heisenberg spin exchange between spin-labeled Na^+,K^+-ATPase [ATP phosphohydrolase (Na^+/K^+-transporting), EC 3.6.1.37] proteins has been studied by saturation transfer ESR spectroscopy in reconstituted membranes. Na^+,K^+-ATPase from the salt gland of *Squalus acanthias* was solubilized in a polyoxyethylene ether detergent, octa(ethylene glycol) dodecyl monoether. Part of the solubilized enzyme was covalently spin-labeled with a nitroxide derivative of indanedione and recombined with various proportions of the unlabeled enzyme while the native lipid/protein ratio was maintained. Purified membranes were then reconstituted from the various samples by precipitation with divalent ions. The reciprocal integrated intensities of the saturation transfer ESR spectra were found to increase linearly with the fraction of protein that was spin-labeled, and the gradient of the concentration dependence increased with increasing temperature over the range 4°–25°C. Comparison with theoretical analyses of the effects of weak Heisenberg spin exchange [Marsh, D. & Horváth, L. I. (1992) *J. Magn. Reson.* 97, 13–26] suggests that the effects on the saturation transfer ESR intensity are attributable to short-range diffusional collisions between the spin-labeled protein molecules. The effective value of the local translational diffusion coefficient is 1.8–2.9 $\mu m^2 \cdot s^{-1}$ at 15°C, depending on the diffusion model used, which is much larger than the values obtained for the long-range diffusion coefficient in cells by photobleaching techniques. The temperature dependence of the translational diffusion is larger than expected but correlates with the anomalous temperature dependence of the rotational diffusion observed in the same system.**

The compositional heterogeneity of biological membranes, with respect to both the lipid and the protein components, suggests the possible existence of functionally differentiated lateral membrane domains (e.g., see refs. 1 and 2). Such a spatial microheterogeneity can be detected by comparing the long- and short-range translational diffusion of integral components within the plane of the membrane. Long-range translational diffusion of integral proteins in membranes has been studied extensively by fluorescence photobleaching techniques (for review see ref. 3). In cells, long-range protein diffusion has been found, in many cases, to be highly restricted, and fluorescence recoveries after photobleaching are often incomplete (e.g., see ref. 1). Photobleaching methods are limited in the lower distance scale to which they may be applied, but techniques that are based on the measurement of bimolecular collision rates seem particularly suited to the study of local translational diffusion. Measurements of Heisenberg spin exchange frequencies are well established for determining short-range translational diffusion of spin-labeled lipid molecules in membranes by ESR spectroscopy (for review see ref. 4). However, conventional ESR techniques are not sufficiently sensitive to low exchange frequencies to be of general use for studying the slower translational diffusion of large integral proteins in membranes.

Saturation transfer ESR (STESR) spectroscopy has been shown to be sensitive to weak spin exchange interactions (5, 6), and this property has been exploited in determining the (slow) translational diffusion rates of proteins in homogeneous solution (7). Here, we investigate the use of the STESR method for the determination of local protein translational diffusion rates in membranes. Covalently spin-labeled Na^+,K^+-ATPase (EC 3.6.1.37) from *Squalus acanthias* is reconstituted with various amounts of the unlabeled Na^+,K^+-ATPase at a fixed total protein/lipid ratio in order to vary the spin concentration while maintaining a constant protein mobility in the membrane. A spin label is used that exhibits little segmental motion when attached to the Na^+,K^+-ATPase (8) and hence maximizes the sensitivity to spin–spin interactions. The concentration dependence of the spin–spin interactions is determined from the integrated intensity of the STESR spectrum and is shown to be sensitive to temperature. Estimates of the translational diffusion coefficient from the effective bimolecular collision rates yield values comparable to those found by photobleaching for integral proteins reconstituted at high dilution in lipid bilayers but are much faster than those found for the long-range translational diffusion of the Na^+,K^+-ATPase in a cellular system.

MATERIALS AND METHODS

Membrane-bound Na^+,K^+-ATPase from the rectal gland of *S. acanthias* was prepared as described previously (9), but omitting the treatment with saponin. The specific activity was ≈1500 μmol ATP hydrolyzed per mg protein per hr (10). Class I sulfhydryl groups in the Na^+,K^+-ATPase membranes were blocked with *N*-ethylmaleimide (NEM) under conditions where the Na^+,K^+-ATPase activity is unaffected (8, 11). The class II sulfhydryl groups, which are essential for the overall Na^+,K^+-ATPase activity, were then labeled selectively as described (8, 11) either with 0.2 mM NEM or with 0.05 mM 2-[(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)-methenyl]indane-1,3-dione (5-InVSL) spin label, which was prepared according to ref. 12 and kindly provided by K. Hideg of the University of Pécs, Hungary.

Labeled membranes were solubilized with octa(ethylene glycol) dodecyl monoether ($C_{12}E_8$) at a detergent/protein ratio of 1.3:1 (wt/wt), whereby only the Na^+,K^+-ATPase is solubilized (13). Solubilized enzyme prelabeled with NEM on class I groups and spin-labeled with 5-InVSL on class II

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Abbreviations: STESR, saturation transfer ESR; 5-InVSL, 2[(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methenyl]indane-1,3-dione; NEM, *N*-ethylmaleimide.

‡To whom reprint requests should be addressed.

groups was then mixed with solubilized enzyme labeled with NEM on both class I and class II groups to give protein weight ratios of (5-InVSL)-labeled enzyme to total enzyme of between 0.23 and 1.0. The solubilized mixture of (5-InVSL)-labeled and NEM-labeled enzyme was precipitated by addition of 10 mM $MnCl_2$ at 0°C, and after 90 min of incubation at 0°C, the re-formed membranes, consisting of Na^+,K^+-ATPase protein and lipids only, were collected by centrifugation, washed three times in 20 mM histidine/25% (vol/vol) glycerol at 10°C, and stored at −20°C. Protein and lipid phosphate were assayed according to refs. 10 and 14, respectively.

Samples for ESR spectroscopy were prepared as described (15). The membrane pellet was freed from excess buffer, taken up into a 1-mm-diameter glass capillary, and trimmed to a sample length of 5 mm (16). Conventional (V_1-display) and saturation transfer (V'_2-display) ESR spectra were recorded as described (8, 15). (V_1 is the first harmonic ESR absorption signal detected in phase with respect to the field modulation. V'_2 is the second harmonic ESR absorption signal detected 90° out of phase with respect to the field modulation.) Standardized sample geometry and spectrometer settings and calibrations were employed (16, 17). Integrals of the STESR spectra, normalized with respect to the intensity of the V_1-mode spectra, were evaluated as described (18). Calibrations of the diagnostic STESR lineheight ratios (L''/L and H''/H) and normalized integral intensities (I_{ST}), in terms of the rotational correlation times of spin-labeled hemoglobin, were taken from ref. 19.

RESULTS AND DISCUSSION

To detect collisionally controlled spin–spin interactions by STESR spectroscopy, the Na^+,K^+-ATPase from shark was labeled selectively on class II sulfhydryl groups with the 5-InVSL spin label. A large batch of Na^+,K^+-ATPase prelabeled with NEM on class I sulfhydryl groups was labeled on class II sulfhydryl groups with 5-InVSL (see *Materials and Methods*), in order to ensure a uniform degree of spin-labeling of the protein. In this way, any intramolecular spin–spin interactions were maintained constant (see ref. 5). The spin-labeled protein was solubilized in $C_{12}E_8$ and various amounts were mixed with solubilized Na^+,K^+-ATPase from the same enzyme batch, which had been both prelabeled on class I sulfhydryl groups and labeled on class II sulfhydryl groups with NEM, to give samples of identical protein/lipid ratio but with differing proportions of spin-labeled protein. The solubilized samples were then reconstituted to yield membranous samples whose sole protein was the Na^+,K^+-ATPase. Lipid phosphate and protein assays confirmed that all samples had very similar lipid/protein ratios. The double-integrated intensity of the conventional ESR spectra (V_1-display) was also found to be linearly proportional to the content of spin-labeled protein in the various samples.

STESR spectra (V'_2-display) of the samples containing different proportions of the spin-labeled protein were recorded at 15°C and are given in Fig. 1. The lineshapes of the STESR spectra are all very similar, confirming that the rotational mobility of the spin-labeled protein is practically identical for all samples. This is as expected, since the lipid/protein ratios are the same, as are the extents of spin-labeling of those proteins that are spin-labeled. STESR spectra recorded at various temperatures indicate that the rotational mobility of the spin-labeled protein increases with increasing temperature, as found previously for samples with a fixed spin-label concentration (8, 15).

The spectra shown in Fig. 1 are all normalized to the same lineheight and therefore do not reflect the differences in the relative intensities. The dependence of the normalized intensities of the STESR spectra, I_{ST}, on the fraction of spin-labeled protein is given in Fig. 2. It is seen that the dependence of the reciprocal saturation transfer integral on the spin concentration in the sample is approximately linear and becomes more pronounced with increasing temperature. This result is consistent with a diffusion-controlled Heisenberg spin exchange interaction taking place between the spin-labeled proteins in the reconstituted membranes (see refs. 6 and 7). Heisenberg spin exchange is expected to have little effect on the STESR lineshapes, as is found in Fig. 1, and to affect only the integrated intensity of the STESR spectrum, as is found in Fig. 2 (6).

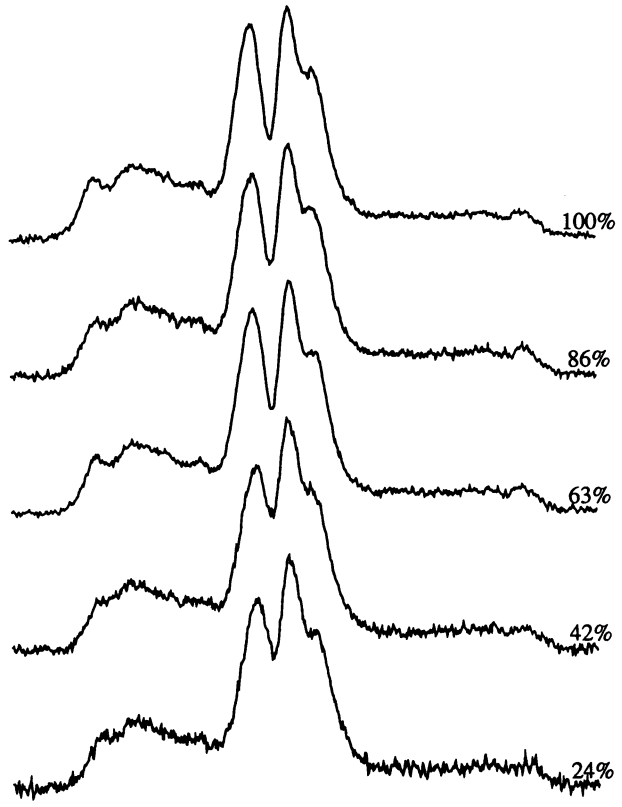

FIG. 1. Second harmonic, 90° out-of-phase absorption STESR spectra (V'_2-display) of (5-InVSL)-labeled class II sulfhydryl groups on Na^+,K^+-ATPase in reconstituted membranes at 15°C. The proportion (%) of the total Na^+,K^+-ATPase that is spin-labeled is indicated for each spectrum. The lipid/protein ratio is 1.2 μmol of lipid phosphate per mg of protein in each case. Total scan width = 10 mT.

If the STESR integral intensity, I_{ST}, is directly proportional to the effective spin-lattice relaxation time, T_1 (20), the dependence of I_{ST} on spin concentration, c, for a powder-pattern lineshape in the presence of Heisenberg spin exchange interaction is given by (6, 7)

$$1/I_{ST} = (1/I^\circ_{ST})(1 + k_{ex}T^\circ_1 \cdot c), \quad [1]$$

where I°_{ST} and T°_1 are the (constant) values of I_{ST} and T_1 in the absence of spin exchange, and k_{ex} is the second-order rate constant for spin exchange. This equation is valid for low concentrations or low exchange rates and is appropriate to the present measurements. The data given in Fig. 2 conform reasonably well to the dependence on spin concentration predicted by Eq. 1. The second-order rate constant for spin exchange is further related to the bimolecular collision rate constant, k_{coll}, between proteins by the intrinsic probability of exchange on collision, p_{ex}, and the normalized cross section for collision between the spin-labeled groups on the protein, σ:

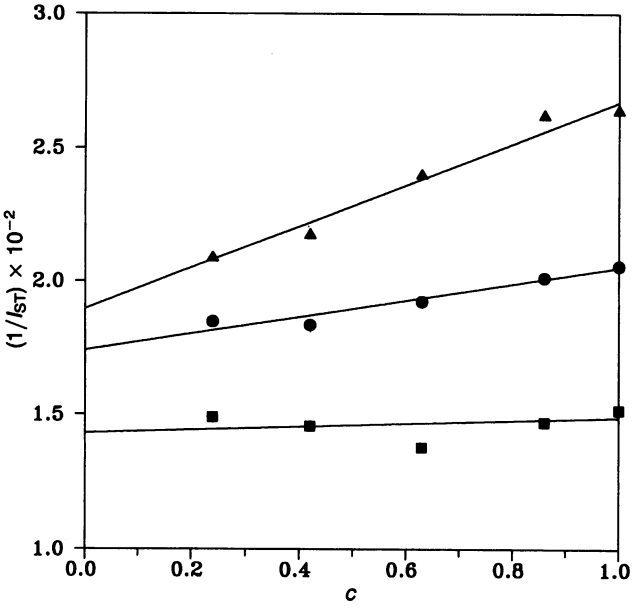

FIG. 2. Dependence of the reciprocal STESR integral intensity, $1/I_{ST}$, on mole fraction, c, of Na^+,K^+-ATPase spin-labeled with 5-InVSL on class II sulfhydryl groups relative to total Na^+,K^+-ATPase in reconstituted membranes. All samples have the same lipid/protein ratio, 1.2 μmol of lipid phosphate per mg of protein. Data were recorded at 4°C (■), 15°C (●), and 25°C (▲). Full lines are linear regressions according to Eq. 1, which yield values of $k_{ex}T_1^* = 0.048$, 0.178, and 0.409 and $I_{ST}^* = 0.710 \times 10^{-2}$, 0.574×10^{-2}, and 0.528×10^{-2} at 4°C, 15°C, and 25°C, respectively.

$$k_{ex} = p_{ex}\sigma k_{coll}, \quad [2]$$

where $p_{ex} = \frac{1}{2}$ for strong exchange (21) and $\sigma \leq 1$, depending on the accessibility of the spin-labeled groups.

The rate constants for exchange obtained from the linear regressions in Fig. 2 are expressed in terms of relative concentrations corresponding to the mole fraction of the protein that is spin-labeled. They must be converted to area fractions relative to the total membrane surface area, which are appropriate units for analyzing the dynamics of diffusional collisions (5). The Na^+,K^+-ATPase protein is composed of an α and a β subunit and is probably present in the membrane as an $(\alpha\beta)_2$ diprotomer. Taking the measured lipid/protein ratio of 350 mol of lipid per mol of $(\alpha\beta)_2$ dimer, an area per lipid molecule of 0.6 nm^2, and the extramembranous dimensions of the $(\alpha\beta)_2$ Na^+,K^+-ATPase dimer of 8 × 6 nm (22) yields values of $k_{coll}\sigma = 0.5 \times 10^6$, 1.9×10^6, and 4.4×10^6 s^{-1} at 4°C, 15°C, and 25°C, respectively. These values for the collision rate constant have been calculated by assuming a value of $T_1^* = 1$ μs (20). The temperature dependence of the calculated values is rather steep, but it is known that the rotational diffusion studied by STESR has an anomalous temperature dependence in this system, evidencing irreversible protein aggregation at higher temperatures (8, 15). This could suggest an unusually high temperature dependence of the membrane viscosity, which would affect also the translational diffusion of the protein.

The bimolecular collision rate constants may be related to the translational diffusion coefficient, if a (hexagonal) lattice model is assumed (e.g., see ref. 4):

$$D_T = (\lambda^2/24)\cdot k_{coll}, \quad [3]$$

where λ is the root-mean-square displacement of the protein in a single diffusive step. This lattice model is admittedly an oversimplification for protein diffusion, but it should give the correct order of magnitude with appropriate choice of the characteristic length, λ. Values for the translational diffusion coefficient of the Na^+,K^+-ATPase obtained for a value of $\lambda \approx 6$ nm (22) are then 0.8, 2.9, and 6.6 $\mu m^2 \cdot s^{-1}$ at 4°C, 15°C, and 25°C, respectively. These values were calculated by assuming that $\sigma \approx 1$, which is consistent with the relatively high degree of labeling of the protein and the surface location of many of the labeled groups (see ref. 23).

An alternative method for deducing the translational diffusion coefficient from the collision rate constant involves solution of the diffusion equation. In two-dimensional systems this gives rise to a collision rate that depends on the time of observation, t, which in the present case must be appreciably less than the intrinsic spin-lattice relaxation time, T_1^*, in order to give rise to observable changes in the STESR integral. For Smoluchowski boundary conditions, the approximate solution at short times (which is appropriate to the present case) is given by (24)

$$k_{coll} = 2\pi D_T[1 + 2d_c(\pi D_T t)^{-1/2}], \quad [4]$$

where d_c is the collision diameter, which is of the order of the protein diameter. For this equation, concentrations must be expressed per unit area, rather than per area fraction. With the data given above, the corresponding values of the collision rate constant become $k_{coll}\sigma = 25$, 93, and 212 $\mu m^2 \cdot s^{-1}$ at 4°C, 15°C, and 25°C, respectively. Taking $t \approx 0.5T_1^*$, $\sigma \approx 1$, and $d_c = 6$ nm then yields estimates for the protein translational diffusion coefficient, $D_T = 0.2$, 1.8, and 7.7 $\mu m^2 \cdot s^{-1}$ at 4°C, 15°C, and 25°C, respectively. These values are similar to those obtained above with very different assumptions by using the lattice model. Although there is some uncertainty with regard to the exact choice for the observation time, t, the overall order of magnitude of the diffusion coefficients seems reasonably well established.

For comparison with the present results, estimates of the translational diffusion coefficient from the hydrodynamic theory of Saffmann and Delbrück (25) are *ca.* 0.9 and 3.5 $\mu m^2 \cdot s^{-1}$ for membrane viscosities of 5 and 1 P (1 P = 0.01 Pa·s), respectively, with relatively little dependence on the intramembranous protein diameter over the range 3–6 nm. Experimental values obtained for the long-range translational diffusion coefficients measured by photobleaching for various integral proteins reconstituted in fluid lipid bilayers are *ca.* 2.3 $\mu m^2 \cdot s^{-1}$ for the acetylcholine receptor in dimyristoyl phosphatidylcholine at 36°C (26), and *ca.* 1.8 $\mu m^2 \cdot s^{-1}$ for both band 3 (erythrocyte anion transporter) in dimyristoyl phosphatidylcholine at 35°C (27) and Ca^{2+}-ATPase in sarcoplasmic reticulum lipids at 36°C (28). These values are also of a similar magnitude to the present measurements for the short-range diffusion of the Na^+,K^+-ATPase, as might be expected, since at high dilution the long-range diffusion in pure lipid bilayers should be relatively unrestricted, which is consistent with complete fluorescence recoveries being obtained after photobleaching. By contrast, in photobleaching experiments on Na^+,K^+-ATPase from Madin–Darby canine kidney cells, only 50% of the labeled protein exhibited long-range lateral mobility, with a translational diffusion coefficient of 0.05 $\mu m^2 \cdot s^{-1}$ (29). Comparison with the present results suggests that there is a considerable difference between the long-range and local translational diffusion of the Na^+,K^+-ATPase protein in this bipolar cellular system, although it should be noted that the translational diffusion of a lipid probe was also rather slow ($D_T \approx 0.2$ $\mu m^2 \cdot s^{-1}$) in the same system.

The STESR method described in the present paper therefore yields local translational diffusion coefficients in a reconstituted system that are in the same range as those obtained with the fluorescence recovery technique in reconstituted membranes. The present method is applicable also to intact cells, provided that the spin-label concentration can be

controlled as is done here with the reconstituted system. Potential candidates for useful spin-labeled ligands include specific antibodies as a general tool and, as in the case of the Na^+,K^+-ATPase, a specific ligand such as spin-labeled ouabain. The STESR method can thus potentially be used to describe molecular motion over short distances, where currently available methods such as the fluorescence recovery technique are not applicable.

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