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A novel spin-label for study of membrane protein rotational diffusion using saturation transfer electron spin resonance. Application to selectively labelled Class I and Class II -SH groups of the shark rectal gland Na^+/K^+-ATPase

Mikael Esmann [1], H. Olga Hankovszky [2], Kálmán Hideg [2] and Derek Marsh [3]

[1] *Institute of Biophysics, University of Aarhus, Aarhus (Denmark),* [2] *Central Laboratory for Chemistry, University of Pécs, Pécs (Hungary) and* [3] *Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, Göttingen (F.R.G.)*

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Na^+/K^+-ATPase in membranous preparations from the rectal gland of *Squalus acanthias* has been spin-labelled either on Class I -SH groups, which maintain overall ATPase activity, or on Class II -SH groups, for which only phosphorylation activity is preserved. Labelling of the Class I groups requires solubilization of the membranes and subsequent reconstitution by precipitation with Mn^{2+} in order to remove contaminating peripheral proteins, which are also labelled. Control experiments with preparations in which the Class II groups are labelled demonstrate that the mobility and aggregation state of the enzyme in the reconstituted membranes are similar to those in the native membrane. Both the conventional maleimide nitroxide derivative and a new benzoylvinyl nitroxide derivative have been used for the labelling. The segmental mobility of the labels and the overall rotational diffusion of the labelled protein have been investigated using saturation transfer ESR spectroscopy. The benzoylvinyl spin-label derivative offers particular advantages for the study of the protein rotational mobility in that the segmental mobility is considerably reduced relative to that observed with the maleimide derivative. This is especially the case for the Class I groups, where the maleimide label exhibits pronounced segmental mobility. Comparison of the results from the two labels indicates that the integral of the saturation-transfer spectrum is much more sensitive to segmental motion than are the diagnostic lineheight ratios. This fact allows a better level of discrimination between the two types of motion. The results from the benzoylvinyl nitroxide-labelled Class I groups suggest that the Na^+/K^+-ATPase is probably present as an $(\alpha\beta)_2$-diprotomer (or higher oligomer) in the native membrane.

Introduction

Na^+/K^+-ATPase is an integral membrane protein responsible for the active transport of Na^+ and K^+ across the plasma membrane of the cell. The present paper is concerned with the oligomeric structure of the protein in the membrane-bound state.

The enzyme consists of α- and β-subunits of molecular mass 112 and 35 kDa, respectively, in equimolar amounts (see Ref. 1 for an overview). The oligomeric structure of the membrane-bound enzyme has not yet been elucidated. The enzyme forms two-dimensional crystals with unit cells consisting of $\alpha\beta$-protomers, $(\alpha\beta)_2$-diprotomers or $(\alpha\beta)_4$-tetraprotomers, depending on the ligands present during crystallization [2–4]. This diversity in oligomeric structure has also been observed with detergent-solubilized preparations, with the oligomeric state and enzymatic activity being determined both by the ligands present and by the detergent/protein ratio [5]. At present there is no conclusive evidence for a single oligomeric structure representing the enzyme species responsible for the active transport of Na^+ and K^+. For a number of years, kinetic evidence has been gathered which suggests that under some conditions there must be an interaction between two α-subunits, each bearing an ATP or ouabain binding

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $C_{12}E_8$, octaethyleneglycol dodecyl monoether; NEM, *N*-ethylmaleimide; 5-MSL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy; BzVSL, 1-oxyl-2,2,5,5-tetramethyl-3-(2-benzoylethenyl)pyrroline; 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.

Correspondence: D. Marsh, Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-3400 Göttingen, F.R.G.

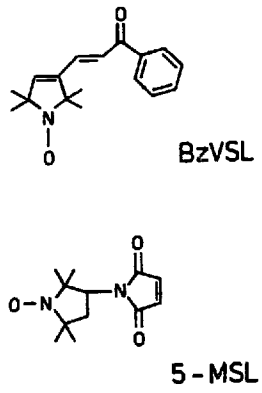

Fig. 1. Structures of the spin labels used for the covalent labelling of Na^+/K^+-ATPase.

site [6,7]. This line of research suggests that the minimum oligomeric structure is an $(\alpha\beta)_2$-diprotomer.

In a previous report, STESR has been used to study the rotational mobility of the covalently spin-labelled Na^+/K^+-ATPase [8]. The results obtained suggested a rotational correlation time in the range 20–50 μs, consistent with an $(\alpha\beta)_2$-structure for the membranous spin-labelled enzyme. In the present paper we extend these studies in two ways. Firstly, we have been able to spin-label the enzyme covalently under conditions where activity is preserved. This way done by labelling only the Class I -SH groups [9,10], followed by a further purification step and reconstitution. Previously, the Class II -SH groups were labelled, which inhibits overall ATPase activity, with only the ability to phosphorylate being retained [11]. Secondly, we have used a novel sulphydryl-reactive label, a benzoylvinyl nitroxide derivative BzVSL (see Fig. 1). This label has been found to have the advantage over the conventional maleimide nitroxide derivative, 5-MSL (Fig. 1) in that the independent segmental motion of the probe relative to the protein is very much reduced. This represents a very significant methodological improvement, since it is found that the maleimide-labelled Class I groups display even greater segmental mobility than do the Class II groups. Hence, the combination of the new labelling procedure and the new spin-label reagent provides an active preparation suitable for study of the rotational diffusion of the membranous Na^+/K^+-ATPase by STESR.

Materials and Methods

Spin-labelled maleimide 5-MSL was obtained from Syva (Palo Alto, CA). BzVSL was synthesized as described by Hankovszky et al. [12]. NEM and glutaraldehyde (grade I) were from Sigma (St. Louis, MO). $C_{12}E_8$ was from Nikko Chemicals (Tokyo, Japan). Na^+/K^+-ATPase-rich membranes from the rectal gland of *Squalus acanthias* were prepared as described by Skou and Esmann [13], but omitting the treatment with saponin. The Na^+/K^+-ATPase constituted typically 50–70% of the protein (determined as the content of α- and β-subunits from SDS gel electrophoresis), and the specific activity ranged accordingly from 1100 to 1500 μmol ATP hydrolysed/mg protein per h. Since solubilization of these preparations in the detergent $C_{12}E_8$ yields an insoluble precipitate, presumably of peripheral proteins, and a supernatant, which contains practically pure Na^+/K^+-ATPase [14], it can be assumed that the Na^+/K^+-ATPase is the only integral protein in the membrane. Na^+/K^+-ATPase activity and protein content were determined as previously described [15].

Class I -SH groups in the Na^+/K^+-ATPase membranes were labelled with BzVSL or 5-MSL, under conditions where the Na^+/K^+-ATPase activity is unaffected, using the following method. Na^+/K^+-ATPase membranes (2–10 mg/ml) were incubated in 30 mM histidine (pH 7.4 at 37°C) in the presence of 150 mM KCl/5 mM CDTA and 35% glycerol (v/v), either with 0.1 mM 5-MSL (incubation time 45 min at 23°C) or with 1 mM BzVSL (incubation time up to 90 min at 37°C, see Fig. 2). The reaction was quenched by addition of 1 mM 2-mercaptoethanol, and the membranes were freed from the reaction mixture by centrifugation three times in 20 mM histidine (pH 7.0 at 20°C) and 25% (v/v) glycerol at 200000 × g. The extent of the inactivation of the Na^+/K^+-ATPase activity was determined from aliquots removed during the incubation procedure (see Fig. 2). Since both the Na^+/K^+-ATPase and contaminating membrane proteins are labelled in this procedure, it was necessary to further purify the Na^+/K^+-ATPase preparation before the ESR measurements. This was done essentially as described earlier [16]. Spin-labelled membranes were solubilized with $C_{12}E_8$ at a detergent-to-protein ratio of 1:1 (w/w), whereby only the Na^+/K^+-ATPase is solubilized [14]. The solubilized protein was precipitated by addition of 10 mM $MnCl_2$ at 0°C, and after 90 min incubation at 0°C, the reformed membranes, consisting of active Na^+/K^+-ATPase protein and lipids only, were collected by centrifugation, washed and used for the ESR experiments.

Prelabelling of Na^+/K^+-ATPase with NEM to block Class I -SH groups and sulphydryl groups in the contaminating proteins in the membrane preparations was performed as follows: Na^+/K^+-ATPase (approx. 1 mg/ml) was incubated at 23°C with 0.1 mM NEM in 30 mM histidine (pH 7.0 at 23°C)/5 mM CDTA/150 mM KCl and 35% (v/v) glycerol for 60 min. The reaction was stopped by addition of 1 mM 2-mercaptoethanol, and the membranes were washed by centrifugation in 20 mM histidine (pH 7.0 at 20°C) and 25% (v/v) glycerol at 200000 × g. Three centrifugations in 27-ml tubes were sufficient to remove residual reac-

tion medium. The prelabelled enzyme was stored in 20 mM histidine and 25% (v/v) glycerol at −20°C.

Selective spin-labelling of the Class II -SH groups, which are essential for the overall Na^+/K^+-ATPase activity, was done as follows. Prelabelled Na^+/K^+-ATPase (see above) was incubated at 37°C in 30 mM histidine (pH 7.4 at 37°C) in the presence of 150 mM KCl/5 mM CDTA/3 mM ATP (Tris salt), either with 0.1 mM 5-MSL (incubation time 60 min) or with 1 mM BzVSL (incubation time 180 min). The reaction was stopped and the enzyme washed as described above for the spin-labelling of Class I -SH groups. The maximal phosphorylation level is 60–80% of that obtained for control enzyme after incubating under the labelling conditions in the absence of spin label [11].

Samples for ESR spectroscopy were prepared according to the following protocol. 1 mg of membranous spin-labelled protein was diluted in 10–27 ml of the desired buffer (usually 30 mM histidine (pH 7.4 at 37°C)/100 mM NaCl/1 mM CDTA) and the membranes pelleted by centrifugation for 45 min at 100000 × *g*. The pellet was freed from excess buffer, taken up into a 1 mm diameter glass capillary and trimmed to a sample length of 5 mm.

ESR spectra were recorded on a Varian E-12 Century Line 9 GHz spectrometer equipped with nitrogen gas flow temperature regulation. 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 peak-to-peak, at the same microwave power as used for recording the STESR spectra. STESR spectra were recorded in the second harmonic, 90° out-of-phase, absorption mode (V_2' display) with a modulation frequency of 50 kHz and a modulation amplitude of 5 G peak-to-peak. Standardized sample geometry and spectrometer settings and calibrations were employed as in the protocol described in Refs. 17 and 18. Integrals of the STESR spectra, normalized with respect to the intensity of the V_1-mode spectra, were evaluated as described in Ref. 19. Calibrations of the diagnostic STESR lineheight ratios (L''/L and H''/H) and normalized integral intensities, in terms of the rotational correlation times of 5-MSL-labelled haemoglobin, were taken from Ref. 19. Corresponding calibrations for BzVSL were found to be essentially similar (Horváth, L.I., personal communication). Further details of the ESR spectroscopy are given in Ref. 8.

Results and Discussion

Na^+/K^+-ATPase-rich membranes from *S. acanthias* were spin-labelled at 37°C. Either the superficial (Class I) groups were spin-labelled directly in the presence of glycerol, or the superficial groups were first prelabelled with NEM and then the buried (Class II) groups subsequently spin-labelled. With the NEM prelabelling, the Na^+/K^+-ATPase membranes could be used directly, since it has been demonstrated that, under these conditions, only -SH groups on the Na^+/K^+-ATPase molecule itself are spin-labelled [9,10]. When the Class I groups are labelled, the membranes must then be solubilized in $C_{12}E_8$ to remove the non-ATPase peripheral proteins (which are also spin-labelled) as an insoluble precipitate. The purified Na^+/K^+-ATPase is then subsequently reconstituted into membranes by precipitating with Mn^{2+} [16]. The reconstitution procedure is accompanied by only a small loss of activity (about 20–30%, see Ref. 16). Labelling the Class I groups leads to no loss of overall enzymatic activity, but on labelling the Class II groups, Na^+/K^+-ATPase activity is lost and the enzyme retains only the ability to be phosphorylated by the substrate [11]. In the present work, the mobility of these two sets of essential and non-essential -SH groups is compared using the two different types of spin label.

The time-course of inactivation of the Na^+/K^+-ATPase on reaction with BzVSL is given in Fig. 2. In the presence of glycerol, the Class I groups are labelled and there is little or no inactivation with 1 mM BzVSL present, whereas in the absence of glycerol the Class II groups are labelled and about 50% of the activity is irreversibly lost within 3 h at 37°C. Th remaining activity almost certainly corresponds to unlabelled protein. The reactivity of the BzVSL label is considerably lower than that of the maleimide spin label (cf. Refs. 8 and 9), and long incubation times at 37°C are therefore required for labelling.

The conventional, in-phase ESR spectra recorded at 0°C of Na^+/K^+-ATPase labelled either with BzVSL or 5-MSL, are given in Fig. 3. The major portion of the spin label is strongly immobilized on the conventional ESR timescale for all four spectra (see for example Ref. 20). For the BzVSL label, only a small proportion of the labelled groups exhibits a high mobility, as represented by the sharp three-line spectral component, when either

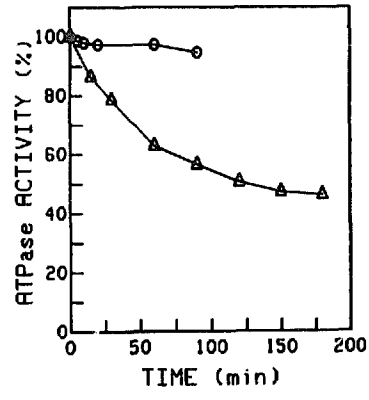

Fig. 2. Time-course of the Na^+/K^+-ATPase activity on labelling the Class I -SH groups (○), or the Class II -SH groups (△) with BzVSL, using the conditions described in the Materials and Methods section.

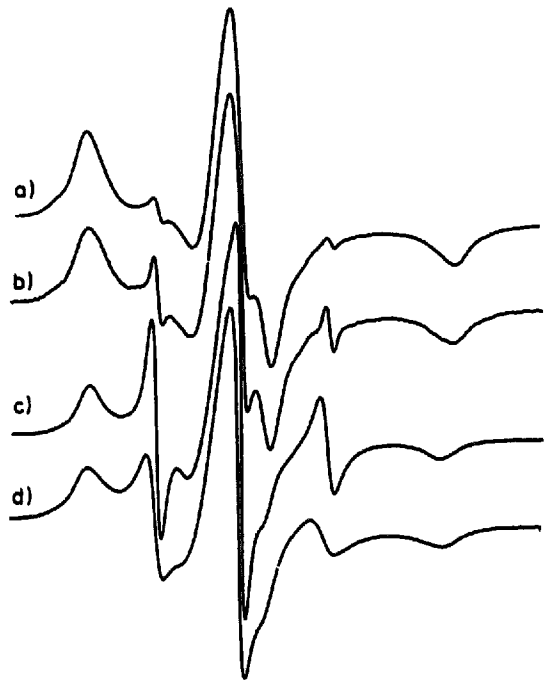

Fig. 3. Conventional ESR spectra (V_1), at 0°C, of Na^+/K^+-ATPase membranes labelled on either the Class I or II -SH groups, with either BzVSL or 5-MSL spin label. (a) BzVSL-labelled Class I groups; (b) BzVSL-labelled Class II groups; (c) 5-MSL-labelled Class I groups and (d) 5-MSL-labelled Class II groups. Total scan width = 100 G.

the Class I or Class II groups are labelled. For the 5-MSL label, the proportion of mobile labelled groups is larger than for BzVSL, but the majority of the spin-label intensity is still associated with the immobilized groups. The degree of immobilization of the motionally restricted component is also greater for the BzVSL label than for the 5-MSL label, as evidenced by the outer hyperfine splitting and outer linewidths [20,21]. For instance, the outer splitting is approx. 69.0 and 70.0 G at 0°C for Class I and Class II groups, respectively, when labelled with BzVSL, whereas the corresponding values with 5-MSL labelling are 66.5 and 67.5 G. Therefore, the conventional spectra already show that the BzVSL label is more rigidly attached to the protein than is the 5-MSL label. Some difference is also seen between the mobility of the Class I and Class II groups, indicating that these are indeed quite distinct groups, as was clear from the different effects on activity.

The second harmonic, 90° out-of-phase, absorption STESR spectra (V_2'-display) of Na^+/K^+-ATPase labelled with BzVSL or 5-MSL on either the Class I or Class II groups are given in Fig. 4. The spectra for the BsVSL spin label, unlike those for 5-MSL, are not appreciably distorted by the sharp mobile spin-label component and the lineheight ratios in the diagnostic STESR regions can be readily measured. Furthermore, the diagnostic lineheight ratios are clearly greater than those for the 5-MSL label, indicating that there is less segmental motion relative to the protein on the STESR timescale for the BzVSL label. The new label therefore reflects more directly the overall rotational diffusion of the protein in the membrane than does the conventional maleimide spin label.

The temperature-dependence of the STESR spectra of the BzVSL-labelled Class I groups is given in Fig. 5. The disturbing influence of the spectrum from the mobile groups becomes greater with increasing temperature, as expected, but the diagnostic lineheight ratios can still be measured over a wide temperature range. As observed previously for the enzyme labelled with 5-MSL on the Class II groups [8], there is an irreversible decrease in the rotational mobility recorded at 0°C, after cycling the temperature to 37°C.

The temperature-dependence of the diagnostic lineheight ratios in both the low-field and high-field spectral regions and the normalized integrated intensity of the STESR spectra are given for both BzVSL and 5-MSL labels and for the labelling of the Class I groups and the Class II groups in Fig. 6. These parameters can be used independently for determining the effective rotational correlation times based on experimental calibrations from spin-labelled haemoglobin in solutions of varying viscosity [19,22]. Qualitatively, the temperature-dependence is similar in all cases and similar to that observed previously for the 5-MSL-labelled Class II groups [8]. Initially there is a decrease in all parame-

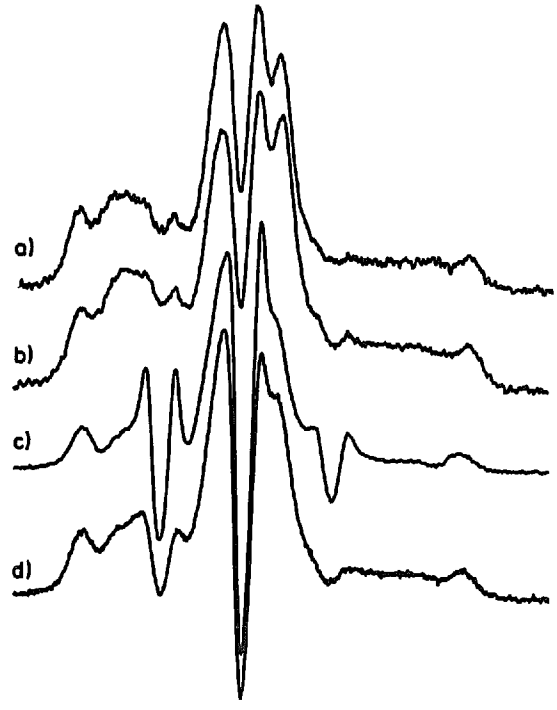

Fig. 4. Second harmonic, 90° out-of-phase STESR spectra (V_2'), at 0°C, of Na^+/K^+-ATPase membranes labelled on either the Class I or II -SH groups, with either BzVSL or 5-MSL spin label. (a) BzVSL-labelled Class I groups; (b) BzVSL-labelled Class II groups; (c) 5-MSL-labelled Class I groups and (d) 5-MSL-labelled Class II groups. Total scan width = 100 G.

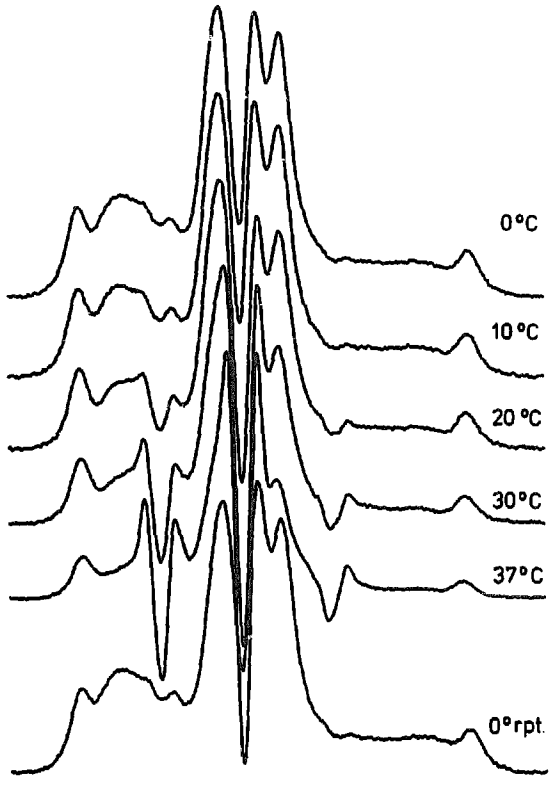

Fig. 5. Temperature-dependence of the second harmonic, 90° out-of-phase STESR spectra (V_2') of Na^+/K^+-ATPase membranes labelled on the Class I -SH groups with BzVSL spin label. The bottom spectrum was recorded at 0 °C after the spectra from 0 to 37 °C were recorded. Total scan width = 100 G.

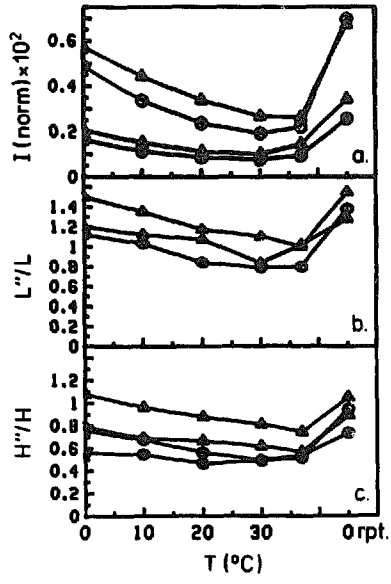

Fig. 6. Temperature-dependence of (a) the normalized integral, (b) the low-field lineheight ratio, L''/L, and (c) the high-field lineheight ratio, H''/H, in the V_2' STESR spectra of Na^+/K^+-ATPase membranes labelled on either the Class I or II -SH groups, with either BzVSL or 5-MSL spin label. (○) BzVSL-labelled Class I groups; (△) BzVSL-labelled Class II groups; (●) 5-MSL-labelled Class I groups; (▲) 5-MSL-labelled Class II groups. The final points on the right-hand side of the plot (0 rpt.) represent repeat measurements at 0 °C after temperature cycling to 37 °C.

TABLE I

Effective rotational correlation times, τ_R^{eff} (μs), deduced from the STESR lineheight ratios, L''/L and H''/H, and the STESR integral intensity for Na^+/K^+-ATPase labelled on the Class I or Class II -SH groups with either 5-MSL or BzVSL spin label [a]

n.d., not determined.

-SH Groups	5-MSL (μs)	BzVSL (μs)
Class I		
L''/L	n.d.	25–100
H''/H	19– 26	20– 50
integral	<1– 1	2– 20
Class II		
L''/L	30–150	60–600
H''/H	30– 60	50–150
integral	<1– 3	3– 35

[a] The range of values given for the effective rotational correlation time corresponds to the maximum and minimum values obtained over the temperature range 0–37 °C. The higher limit corresponds to 0 °C and the lower limit to either 30 or 37 °C.

ters with increasing temperature, corresponding to an increase in rotational mobility. Then, on approaching 37 °C, there is a levelling-off, or small increase in the parameters, which upon returning to 0 °C is reflected by an irreversible increase in rotational mobility relative to the first recording at 0 °C (see Fig. 6). This latter was previously attributed to a time- and temperature-dependent decrease in the rotational mobility, most probably resulting from an irreversible protein aggregation [8]. The effect is larger with the 5-MSL spin label, but this is probably due to the shorter initial incubation time on labelling at 37 °C: 60 min for 5-MSL as opposed to 180 min for BzVSL.

The effective rotational correlation times deduced from the lineheight ratios and normalized integrals, using calibrations from isotropically tumbling spin-labelled haemoglobin, are given for both labels attached to Class I and Class II groups in Table I. BzVSL yields consistently higher values than 5-MSL, indicating that segmental motion is better suppressed on labelling with BzVSL. Note that it was not possible to measure the low-field lineheight ratio, L''/L, when the Class I groups were labelled with 5-MSL, because of the overlap of the spectral component from the mobile groups. The exact nature of the segmental motion, which is sensed by the 5-MSL label and preferentially suppressed with BzVSL, is not precisely known. Nor is it known whether the two labels react with precisely the same sulphydryl groups. However, the overall reactivity pattern with respect to the Class I and Class II -SH groups is preserved between the two labels, strongly suggesting that similar groups are modified by each label. In addition, examination of the chemical structures of the two labels (Fig. 1) reveals that BzVSL has a shorter linkage between the point of attachment and the

nitroxide ring than does 5-MSL. Indeed, the new label was designed with exactly this point in mind, and model studies have shown that the point of nucleophilic attack is at the β-position of the vinyl ketone. Therefore, it seems likely that the segmental motion is predominantly of a local nature and occurs at the point of nitroxide labelling.

In general, the effective correlation times deduced from the Class I groups are somewhat shorter than those obtained from the Class II groups, perhaps indicating a slightly greater segmental flexibility of the attachment to the former groups. However, it is clear that the labelling of the Class I groups with BzVSL yields an active enzyme for which the STESR spectra predominantly reflect the overall rotational mobility of the protein. It will be noted that the labelling of the Class I groups differs from that of the Class II groups due to the solubilization and reconstitution steps required in the former case. Control experiments in which Class II groups were labelled and the labelled enzyme was subsequently subjected to the same solubilization and reconstitution steps as in the Class I group labelling procedure have shown that these additional steps have little or no effect on the rotational mobility of the protein.

The effective rotational correlation times deduced from the normalized STESR integrals are uniformly lower than those deduced from the diagnostic STESR lineheight ratios. However, the discrepancy is less for BzVSL than for 5-MSL, indicating, as suggested previously, that the difference arises from the segmental motion of the label relative to the protein [8]. As also suggested previously, the closer agreement between the effective correlation times for the two labels deduced from the lineheight ratios indicates that the lineheight ratios are less sensitive to segmental motion and therefore can be used as a more reliable indicator of the overall protein rotational diffusion. The central lineheight ratios (see Fig. 4) are also suggestive of shorter correlation times than those deduced from the outer lineheight ratios, which might indicate an anisotropic segmental motion. However, even for the BzVSL label, these ratios are distorted by the underlying spectrum of the component with very rapid motion (cf. also Fig. 3). Therefore, these ratios have not been included in the analysis.

It was previously argued that the STESR lineheight ratios deduced from the 5-MSL-labelled Class II groups give a lower estimate for the rotational mobility of the protein [8]. The present results with the new BzVSL label attached to Class I groups now give a more reliable estimate for the rotational diffusion coefficient of the active enzyme in the membrane because of the reduced segmental motion of this label. Uncertainties still remain with regard to the orientation, θ, of the nitroxide z-axis with respect to the protein rotation axis (cf. Ref. 23). The rotational correlation time of the protein is given approximately by $\tau_{R\,\parallel} = (\tau_R^{eff}/2) \cdot \sin^2 \theta$, where τ_R^{eff} is the effective rotational correlation time deduced from the STESR calibration with the haemoglobin isotropic model system, the values of which are listed in Table I [8,22]. Thus, an upper limit for the rotational correlation time of the protein would be $\tau_{R\,\parallel} = 25$–50 μs at 0 °C, deduced from the values for the BzVSL-labelled Class I groups in Table I. This would correspond to the $\theta = 90°$ orientation of the nitroxide axes. For a reasonable (upper) estimate of the membrane viscosity of $\eta = 5$ P, the rotational correlation time of the Na^+/K^+-ATPase deduced from dimensional information is $\tau_{R\,\parallel} \simeq 10$ μs for the protomer and $\tau_{R\,\parallel} = 34$ μs for the diprotomer [8]. Thus, for the protein to be in the protomeric form would require that all the spin-label groups be oriented within approximately 20° of the principal diffusion axis to be consistent with the STESR data. In view of the multiple labelling sites within the Class I and Class II groups, it therefore seems more likely that the native protein is present as a diprotomer (or possibly a higher oligomer) within the membrane *.

This suggestion of structural oligomerism is in line with functional (kinetic) observations that require two or more interacting protomers. It is not possible to have functional oligomers and structural protomers, whereas the opposite, namely functional protomers and structural oligomers, is indeed possible.

In summary, the Class I groups of the Na^+/K^+-ATPase can be labelled specifically with BzVSL to produce a preparation suitable for studying the mobility and aggregation of the enzyme in a near-native state. Segmental mobility is efficiently suppressed with BzVSL and it seems likely that this label will also offer considerable advantages in the study of other proteins using STESR spectroscopy. Experiments are currently underway to test the generality of these results in different systems and to search for other similar vinyl ketone labels which may have enhanced reactivity and yet retain the favourable characteristics for STESR studies.

* The reliability of the measured correlation times is approx. ±10–15% when obtained from H''/H and ±50% when obtained from L''/L. This variability derives principally from the reproducibility between preparations. Therefore the measured values should be sufficiently sensitive to distinguish between protomer and diprotomer (a factor of 3.4 in the correlation time), especially when using the high-field ratios (cf. Table I). Additional uncertainties arise from the estimate of the effective membrane viscosity, which enters linearly into the theoretically predicted correlation time. For this reason an upper estimate was taken (see Ref. 24). The effective viscosity in Na^+/K^+-ATPase membranes at 0 °C would have to be approx. 2.5-times greater than this upper limit for dimyristoyl phosphatidylcholine bilayers in the fluid phase, if the measured correlation times were to be consistent with a protomer. In view of the different uncertainties, a protomer cannot be completely excluded but the correlation time data tend to favour a diprotomer.

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