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Indanedione spin labelling of Na,K-ATPase

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Abstract

The indanedione series of vinyl ketone spin-labelling reagents has been extended in two ways: by increasing the length of the rigid spacer between the reactive centre and the nitroxide ring, or by introducing an electrophilic substituent (that could also hinder its rotation) at the bridge head position of the nitroxide ring. Three reagents of this new series have been used to spin label the Class II thiol groups of membranous Na,K-ATPase from *Squalus acanthias*. With a conjugated diene spacer, the majority of spin labels are strongly held but a minor population is relatively mobile at 37 °C. With a conjugated triene spacer, the nitroxide is still strongly held but a portion of the label is non-covalently bound. The 4-bromo-pyrroline derivative (with short vinyl spacer) is tightly held at the attachment site, and the conventional electron paramagnetic resonance (EPR) spectra distinguish between the two enantiomeric structures which differ in their mobility at 37 °C. Saturation transfer EPR (ST-EPR) spectra of this label at 4 °C have been used to determine the dependence of the protein rotational mobility on ionic strength. Electrostatic repulsion contributes to the lateral interactions between Na,K-ATPase molecules.

Keywords: Spin label; Electron paramagnetic resonance; Saturation transfer electron paramagnetic resonance; Indanedione; Enantiomer; Na,K-ATPase

1. Introduction

The indanedione class of vinyl ketone spin-labelling reagents is found particularly advantageous for saturation transfer electron paramagnetic resonance (ST-EPR) studies of the rotational diffusion of membrane proteins [1–5] and for orientational studies on muscle proteins by using conventional EPR [6,7]. As part of a development programme with these reagents, we have characterised derivatives that are substituted across the bridge head position of the nitroxide ring (4-pyrroline derivatives) [3]. Here we extend these studies by increasing the length of the rigid link between the vinyl ketone and the nitroxide ring. We also introduce a 4-bromo-pyrroline derivative, the EPR spectra of which distinguish between the two enantiomeric structures in terms of both local mobility and environmental polarity. As previously [2,3], we label Class II-SH groups of membranous Na,K-ATPase selectively by masking fast-reacting groups with *N*-ethyl maleimide (NEM) in the presence of 40% glycerol. These preparations retain the capacity to phosphorylate from ATP.

2. Materials and methods

2.1. Spin label preparation

Scheme 1 gives the chemical structures and scheme for synthesis of the three new indanedione nitroxide derivatives introduced in this study. Syntheses were essentially according to the method described in Hankovszky et al. [8]. The

Abbreviations: 5-InVSL, 2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethylene)-indan-1,3-dione; 5-EtMInVSL, *trans*-4-(1,3-dioxo-indan-2-ylidenemethyl)-1-oxyl-2,2,5,5-tetramethyl-pyrrolidine-3-carboxylic acid ethyl ester; 5-CxMInVSL, *trans*-4-(1,3-dioxo-indan-2-ylidenemethyl)-1-oxyl-2,2,5,5-tetramethyl-pyrrolidine-3-carboxylic acid; 5-BrInVSL, 2-(4-bromo-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethylene)-indan-1,3-dione; 5-(C3)InVSL, 2-[3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-allylidene]-indan-1,3-dione; 5-(C5)InVSL, 2-[5-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-3-methyl-penta-2,4-dienylidene]-indan-1,3-dione; NEM, *N*-ethyl maleimide; EPR, electron paramagnetic resonance; ST-EPR, saturation transfer EPR

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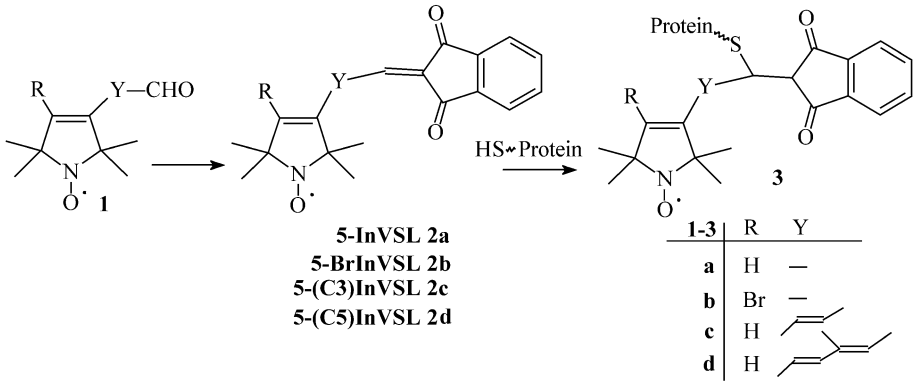

Scheme 1. Chemical structures and reaction scheme for synthesis of the new indanedione pyrroline nitroxide derivatives. Y represents the link between the pyrroline ring and the β-sp^2 carbon (**2a-d**) at which nucleophilic addition (**3**) takes place.

general reaction for synthesis of the various nitroxides was a condensation of a nitroxide aldehyde with appropriate compounds containing an active methylene group, giving α,β,γ,δ-dienes. It has been shown previously that these reagents add to SH groups at the β-carbon atom of the pyrroline-containing diene [8].

Melting points were determined on a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N) were performed on EA 1110 Elemental Analyser apparatus, or (for Hal) were carried out titrimetrically by Schöniger's method. The IR spectra (Specord 85) were in each case consistent with the assigned structure. Mass spectra were recorded on a VG TRIO-2 instrument in the EI mode (70 eV, direct inlet). Flash column chromatography was performed on Merck Kieselgel 60 (0.04–0.063 mm).

2.1.1. Synthesis of spin-labelled 1,3-indandiones (2a–d)

To a solution of indan-1,3-dione (146 mg, 1 mmol) and aldehyde **1a** [9], **1b** [10,11], **1c** [9] or **1d** [12] (1 mmol) in ethanol (10 ml), 10% aqueous NaOH (1 ml) was added and stirred for 3 h at room temperature. The reaction mixture was then acidified with 10% H_2SO_4, extracted with ether (3 × 20 ml), dried ($MgSO_4$) and evaporated. The residue was purified by flash chromatography using hexane/ethyl acetate to give the spin-labelled indan-1,3-diones.

2-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethylene)-indan-1,3-dione (**2a**) (5-InVSL) described earlier [8].

2-(4-Bromo-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethylene)-indan-1,3-dione (**2b**) (5-BrInVSL): Yield: 270 mg (72%); mp: 121–123 °C; Anal. calcd. for C 57.62%, H 4.57%, N 3.73%, Br 21.29%; found C 57.47%, H 4.62%, N 3.81%, Br 21.15%; IR (nujol) *v* (cm^{-1}): 1720, 1690 (C=O); MS (EI) *m/z* (%): 374/376 (M^+,4,4).

2-[3-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-allylidene]-indan-1,3-dione (**2c**) [5-(C3)InVSL]: Yield: 209 mg (65%); mp: 147–148 °C. Anal. calcd. for C 74.51%, H 6.25%, N 4.34%; found C 74.39%, H 6.21%, N 4.42%; IR (nujol) *v* (cm^{-1}): 1715, 1680 (C=O); MS (EI) *m/z* (%): 322 (M^+,8).

2-[5-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-3-methyl-penta-2,4-dienylidene]-indan-1,3-dione (**2d**) [5-(C5)InVSL]: Yield: 246 mg (68%); mp: 157–158 °C. Anal. calcd. for C 76.22%, H 6.67%, N 3.86%; found C 76.38%, H 6.49%, N 3.78%; IR (nujol) *v* (cm^{-1}): 1710, 1680 (C=O); MS (EI) *m/z* (%): 362 (M^+,30).

2.2. Enzyme preparations and labelling

Na,K-ATPase from the rectal gland of *Squalus acanthias* was prepared as described by Skou and Esmann [13], but without the treatment with saponin. The Na,K-ATPase typically constituted 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 hour). Na,K-ATPase activity and protein content were determined as previously described by Esmann [14].

Prelabelling of Na,K-ATPase with NEM to block Class I SH groups and sulfhydryl groups of non-Na,K-ATPase proteins in the membrane preparations was performed as follows (see Ref. [15] for details): 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 36% (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 200,000 × *g*. Three centrifugations in 27-ml tubes were sufficient to remove residual reaction 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 [15]: Prelabelled Na,K-ATPase (see above) was incubated with the required amount of nitroxide-labelled reagent 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). The reaction was stopped by addition of 1 mM 2-mercaptoethanol, and the membranes were washed by

Table 1
Half-times for inactivation of shark Na,K-ATPase by indanedione derivatives at 37 °C in the presence of 150 mM KCl and 3 mM ATP (pH 7.4)

Label	[Label] (μM)	$t_{1/2}$ (min)	$1/([\text{Label}] \times t_{1/2})$ ($\text{mM}^{-1}\ \text{min}^{-1}$)
5-(C3)InVSL	173	3	1.93
5-(C5)InVSL	38	50	0.53
5-BrInVSL	148	3	2.25

centrifugation in 20 mM histidine (pH 7.0 at 20 °C) and 25% (v/v) glycerol at 200,000 × *g*. The spin-labelled enzyme was stored in 20 mM histidine and 25% (v/v) glycerol at −20 °C. The various spin labels were added as dimethylformamide (DMF) or ethanol solutions. The final DMF or ethanol concentration was less than 1%. This concentration of organic solvent in the incubation medium had no effect on enzyme activity.

Extraction with albumin was performed as follows: 1 ml of membranes labelled at Class II groups with 5-(C5)InVSL was mixed with 19 ml of a 30 mM histidine/100 mM NaCl/1 mM CDTA buffer solution containing 154 mg albumin (Behringwerke) and allowed to equilibrate for 60 min at 20 °C. After centrifugation at 100,000 × *g* for 60 min at 4 °C, the pellet was resuspended in 20 ml of the same buffer with 250 mg albumin, equilibrated and centrifuged as above. The resulting pellet—now albumin-extracted twice—was resuspended in 20 ml buffer without albumin and pelleted again by centrifugation. This pellet was taken up in a capillary, trimmed to 5 mm and used for EPR as described in Section 2.3.

2.3. EPR spectroscopy

Samples for EPR spectroscopy were prepared according to the following protocol [16]: 1 mg of spin-labelled protein was diluted in 10 ml buffer [30 mM histidine (pH 7.4 at 37 °C)/100 mM NaCl/1 mM CDTA] and the membranes pelleted by centrifugation at 6 °C for 45 min at 100,000 × *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.

EPR spectra were recorded on a Varian E-12 Century Line 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation. Conventional, in-phase, absorption EPR 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 ST-EPR spectra. ST-EPR 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. Standardised sample geometry and spectrometer settings and calibrations were employed as in the protocol described in Refs. [17,18]. Integrals of the ST-EPR spectra, normalised with respect to the intensity of the V_1-mode spectra, were evaluated as described in Ref. [19]. Calibrations of the diagnostic ST-EPR lineheight ratios (L''/L and H''/H) and normalised integral intensities, in terms of the rotational correlation times of spin-labelled haemoglobin, were taken from Ref. [20]. Further details of the EPR spectroscopy are given in Ref. [16].

3. Results

Spin labelling was followed from the loss in enzyme activity. Table 1 gives the rates at which ATP hydrolysis is inactivated on modification of Class II-SH groups by the different indanedione derivatives. As demonstrated previously [21], inactivation is biphasic. With the 5-BrInVSL derivative, 80% of the activity disappears in the first phase, as for the parent 5-InVSL and in inactivation by maleimide [2,21]. For the two derivatives with longer spacers between nitroxide and indane rings, 5-(C3)InVSL and 5-(C5)InVSL, more than 20% of the activity is retained in the slowly inactivating component.

Fig. 1 gives the temperature dependence of the conventional EPR spectra of the indanedione derivative 5-(C3)InVSL with a conjugated diene (3 C-atom) spacer between the indane and nitroxide rings. Most of the spin labels are rather tightly held, as seen by the large outer hyperfine splitting ($2A_{max}$ = 69.5 G at 4 °C) and only weak

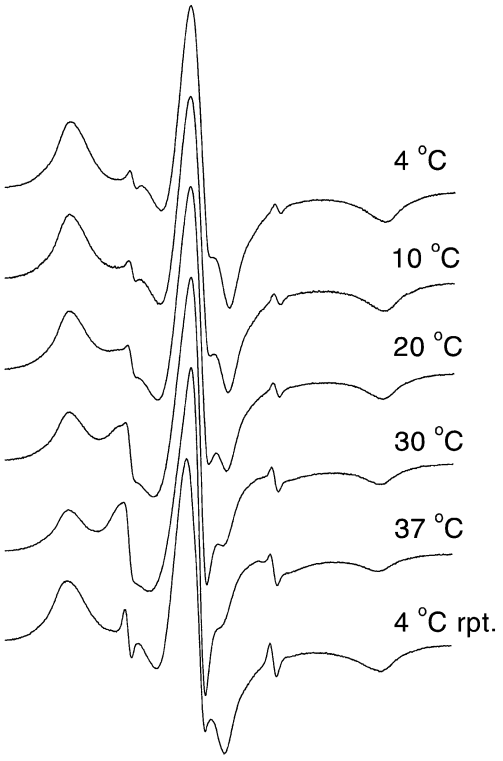

Fig. 1. Temperature dependence of the conventional EPR spectra for Na,K-ATPase spin labelled at Class II SH-groups with the indanedione derivative 5-(C3)InVSL. Total scan width = 100 G.

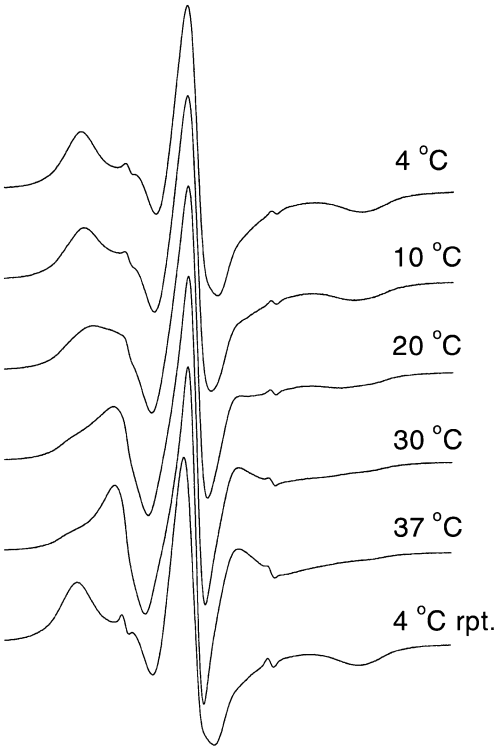

Fig. 2. Temperature dependence of the conventional EPR spectra for Na,K-ATPase spin labelled at Class II SH-groups with the indanedione derivative 5-(C5)InVSL. Total scan width = 100 G.

temperature dependence (see, e.g., Ref. [22]). At 37 °C, a minor proportion of the labelled groups exhibits an isotropic (although differentially broadened) spectrum.

Fig. 2 gives the conventional EPR spectra of the indanedione derivative 5-(C5)InVSL with a conjugated triene (5 C-atom) spacer between the indane and nitroxide rings. The spectra at higher temperatures very obviously consist of two components that are characteristic of very different mobility. Fig. 3 gives the temperature dependence of the EPR spectra from the same spin label after extracting the membranes with albumin. The more mobile spectral component is removed completely, indicating that it represents non-covalently bound spin label, quite possibly associated with the membrane lipid. The covalently bound label again is rather rigidly held, with an outer hyperfine splitting of $2A_{max} = 68.4$ G at 4 °C and little temperature dependence. No isotropic spectral component (other than a vanishingly small amount of free spin label) is evident for this label at 37 °C.

Fig. 4 gives the conventional EPR spectra of the short-link (1 C-atom) indanedione derivative substituted with bromine at the 4-position of the pyrroline ring (5-BrInVSL). The spectra are particularly sharp, as evidenced by the partially resolved superhyperfine structure in the low-field flanks. Nevertheless, the spectra at 4 °C clearly consist of two overlapping powder patterns with somewhat different outer hyperfine splittings. At 37 °C, the outer hyperfine splitting of the inner component decreases markedly and the linewidths of these outer peaks increase. By contrast, the outer component of the low-temperature powder pattern displays relatively little temperature dependence. The two spectral components not only display different mobilities and temperature dependence but also different microwave saturation properties at low temperature. At a microwave power of 40 mW, the outer of the two components is suppressed and the outer hyperfine splitting measured is that of the inner component (see Table 2). In addition, this allows measurement of the linewidths of the outer peaks from the two components. These values are given in Table 2. For both components, the lines are considerably narrower than those of the two other indanedione derivatives. This undoubtedly contributes to the good resolution of these two closely spaced components in the powder patterns at low temperature.

At 4 °C, both spectral components in Fig. 4 are strongly immobilised on the conventional nitroxide EPR timescale. This means that the 5-BrInVSL label should then record the overall rotational diffusion of the membrane-bound ATPase protein. Fig. 5 gives the ST-EPR spectra of the 4-bromo-pyrroline derivative at 4 °C, for Na,K-ATPase membranes suspended in buffer of increasing NaCl concentration. All ST-EPR spectra have very appreciable intensity in the diagnostic regions at low and high field (see, e.g., Ref. [23]), demonstrating that they are registering the overall

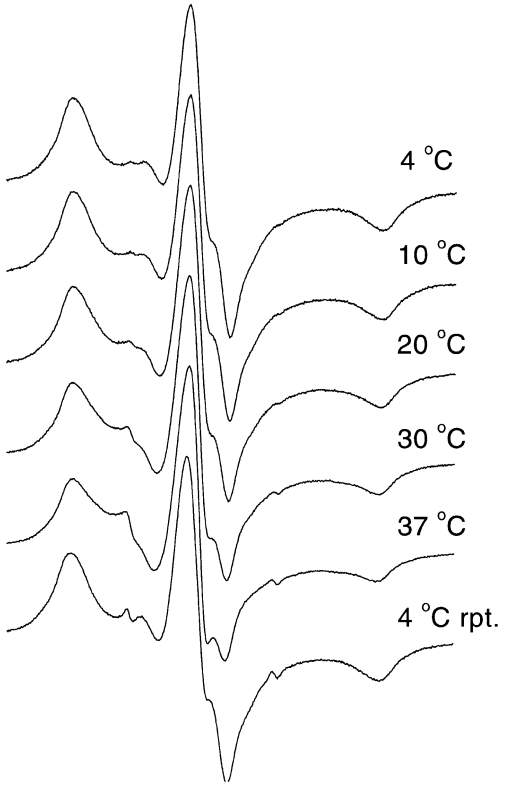

Fig. 3. Temperature dependence of the conventional EPR spectra for Na,K-ATPase spin labelled at Class II SH-groups with the indanedione derivative 5-(C5)InVSL, and subsequently washed twice with albumin. Total scan width = 100 G.

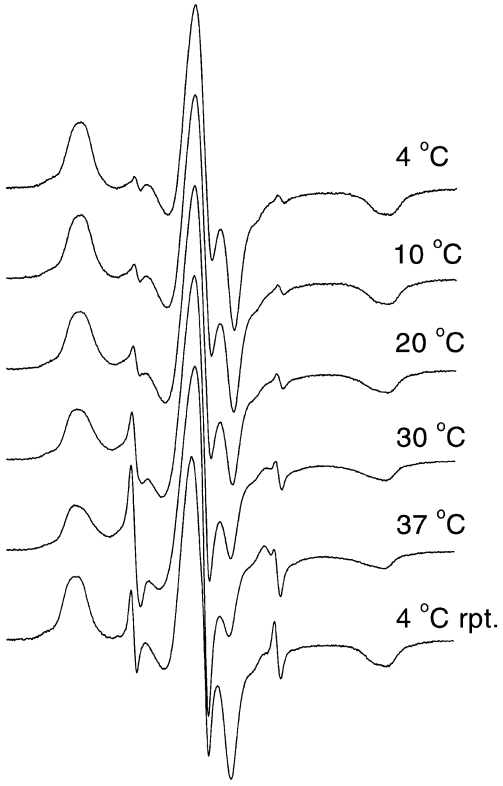

Fig. 4. Temperature dependence of the conventional EPR spectra for Na,K-ATPase spin labelled at Class II SH-groups with the indanedione derivative 5-BrInVSL. Total scan width = 100 G.

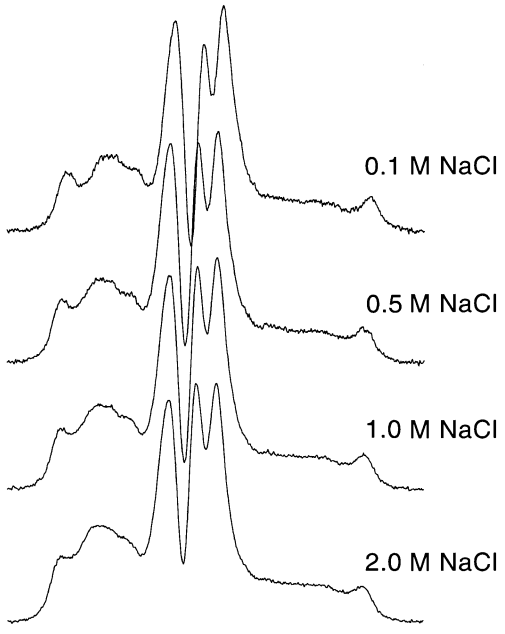

Fig. 5. Second harmonic, 90° out-of-phase absorption ST-EPR spectra (V_2' -display) of 5-BrInVSL spin-labelled Class II SH-groups in Na,K-ATPase at 4 °C. The NaCl concentration (M) of the suspending medium is indicated for each spectrum. Total scan width = 100 G.

rotational diffusion of the protein. Intensities in the diagnostic regions of the ST-EPR spectra increase with increasing ionic strength. This corresponds to a progressive hindering of the rotational mobility of the protein, as is indicated by the effective rotational correlation times. The latter are established from calibrations with isotropically rotating haemoglobin [23] and are given in Fig. 6. In principle, the L''/L and H''/H lineheight ratios reflect the same (anisotropic) motion. The normalised ST-EPR integral contains additionally contributions from motions that affect only the centre of the ST-EPR spectrum: uniaxial rotation

Table 2
Parameters of the outer hyperfine peaks in the conventional EPR spectra recorded at 4 °C for Na,K-ATPase labelled at Class II SH-groups[a]

Label	$2A_{max}$ (G)	ΔH_l (G)	ΔH_h (G)	Ref.[b]
5-(C3)InVSL	69.5	4.1	4.3	1
5-(C5)InVSL (washed)	68.4	4.3	4.6	1
5-BrInVSL (10 mW)	69.9 (outer)	3.3	2.8	1
5-BrInVSL (40 mW)	65.9	3.6	4.6	1
5-InVSL	71.7	2.6	2.9	2
5-EtMInVSL	71.1	2.7	2.9	2
5-CxMInVSL	69.6	2.4	3.2	2

[a] $2A_{max}$ is the outer hyperfine splitting and ΔH_l, ΔH_h are the widths at half-height of the low-field and high-field outer hyperfine peaks, respectively.
[b] References: 1: This work; 2: Ref. [3].

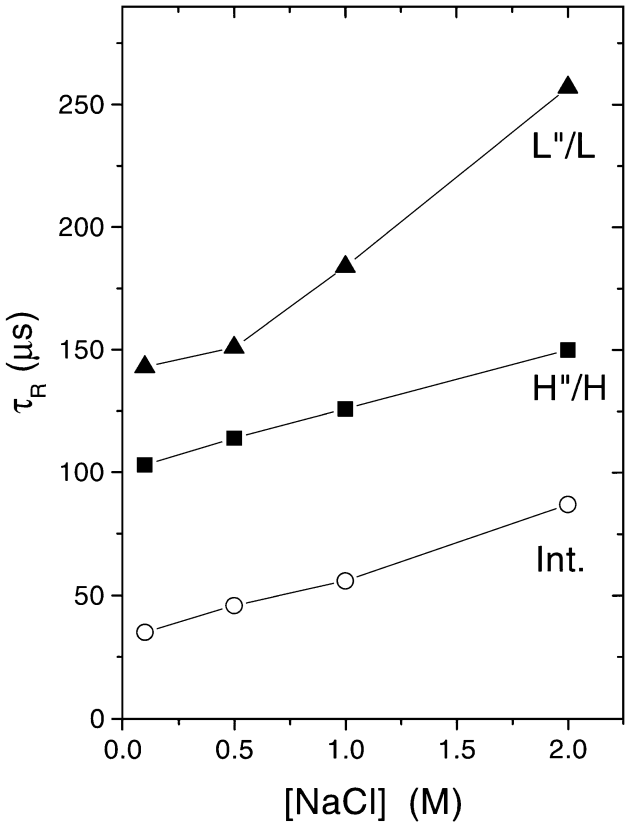

Fig. 6. Ionic strength dependence of the effective rotational correlation times, τ_R, deduced from the low-field (triangles) and high-field (squares) diagnostic lineheight ratios and the integrated intensity (circles) of the ST-EPR spectra of the 4-bromo-indanedione spin label, 5-BrInVSL, at 4 °C.

about the nitroxide *z*-axis and possibly also limited segmental motions. For this reason, the integral parameter yields significantly lower effective rotational correlation times than the outer lineheight ratios.

4. Discussion

The 4-bromo-pyrroline derivative (5-BrInVSL) and that with the conjugated diene spacer [5-(C3)InVSL] have comparably high reactivity to Class II-SH groups of the Na,K-ATPase (see Table 1). Only for the 5-(C5)InVSL derivative with the conjugated triene spacer is the inactivation rate very considerably slower. Possibly this is due in part to the partitioning of the more hydrophobic derivative into the lipid (see above and Fig. 2). This would reduce the concentration of label available for reaction with the −SH group(s) that are essential for activity.

The two spectral components obtained with the 4-bromo-pyrroline derivative, 5-BrInVSL, are of approximately equal intensity. By analogy with similar spectra from antibody-bound spin-labelled haptens with pyrrolidine rings [24–26], the two components are attributed to the two optical enantiomers. In the case of the indanedione/vinyl ketone pyrroline derivatives, the chiral centre is generated at the β-carbon by reaction with the −SH group on the protein. Bromine substitution at C-atom 4 appears to enhance the effects of this chirality, producing well-resolved EPR spectra of the two optical enantiomers. The spectral differences that are evident in Fig. 4 indicate that the environment of the attached nitroxide differs for the enantiomers, both in terms of the local segmental mobility and possibly also the local polarity [25].

Table 2 gives the outer hyperfine splittings of the different labels, including the two enantiomers of the 4-bromo derivative 5-BrInVSL. If it is assumed that the mobility of all labels is low at this temperature, the differences in outer splitting can be attributed (at least in part) to differences in polarity of the local environment of the nitroxide (see, e.g., Ref. [22]). To this list may be added previous data from the parent indanedione, 5-InVSL, and two other 4-pyrroline derivatives [3]. Conventional EPR spectra of the whole series at 2 °C are shown in Fig. 7. The ranking of the outer hyperfine splittings, and hence also polarity, is in the order: 5-InVSL>5-EtMInVSL>5-BrInVSL (outer)>5-CxMInVSL>5-(C3)InVSL>5-(C5)InVSL>5-BrInVSL (inner). The diene- and triene-linked derivatives [5-(C3)InVSL and 5-(C5)InVSL] place the nitroxide progressively further away from the cysteine side chain to which the indanedione is attached. For cysteines that are in the transmembrane section of the protein, this might place the nitroxide deeper into the hydrophobic lipid phase of the membrane. Alternatively, the polarity difference between the enantiomers of a spin-labelled dinitrophenol hapten has been interpreted in terms of the proximity to a charged residue [25]. Removal of the nitroxide from the vicinity of a charged group therefore might also contribute to the difference between the two enantiomers. It should be noted that outer hyperfine splittings at 2 °C may still contain some contribution from limited-amplitude librational motions, additional to that from polarity.

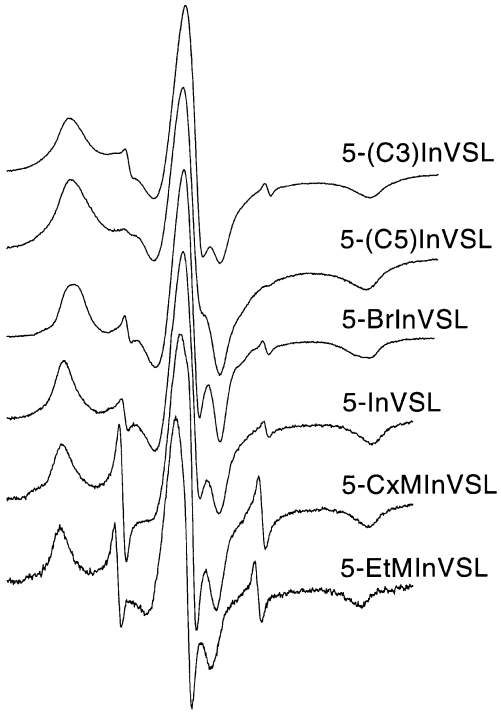

Fig. 7. EPR spectra recorded at 2 °C of shark Na,K-ATPase spin labelled at Class II groups with different nitroxide derivatives. Indanedione derivatives are bound to the protein after labelling for 60 min at 37 °C with 0.4 mM spin label (5-EtMInVSL and 5-CxMInVSL), 0.04 mM for 5-InVSL or after labelling for 30 min with the concentrations indicated in Table 1 for 5-(C3)InVSL, 5-(C5)InVSL and 5-BrInVSL. Total scan width = 100 G.

The dependence of the ST-EPR-determined rotational correlation times on ionic strength (Fig. 6) demonstrates a slowing down in rotation of the membranous Na,K-ATPase, as electrostatic interactions between proteins become shielded by mobile counterions. Additional slowing down may accompany partial dehydration that occurs at high salt concentrations (~ 2 M NaCl), which would serve to reduce repulsive interactions arising from hydration forces (cf. Ref. [27]). By analogy with the dependence on protein concentration of the rotational diffusion rate of cytochrome oxidase reconstituted in phosphatidylcholine [28], the slowing down of Na,K-ATPase rotation is attributed to an increase in frequency of molecular collisions on diminishing the repulsive interactions between protein molecules. Previous ST-EPR measurements on membranous Na,K-ATPase have already demonstrated the occurrence of intermolecular collisions controlled by translational diffusion within the membrane plane [4]. In principle, therefore, ionic strength effects on enzyme activity might in some way be mediated by intermolecular interactions.

Acknowledgements

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