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# Analysis of thiol-topography in Na,K-ATPase using labelling with different maleimide nitroxide derivatives

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Spin-label EPR spectroscopy of shark rectal gland Na,K-ATPase modified at cysteine residues with a variety of maleimide-nitroxide derivatives is used to characterize the different classes of sulphydryl groups. The spin-labelled derivatives vary with respect to charge and lipophilicity, and the chemical reactivity towards modification and inactivation of the Na,K-ATPase is dependent on these properties. Ascorbate is used to reduce the spin-labels in situ, and the kinetics of reduction of the protein-bound spin-labels are found also to depend on the nature of the maleimide-nitroxide derivative. The Na,K-ATPase is labelled either at Class I groups (with retention of enzymatic activity) or at Class II groups (where the enzymatic activity is lost). Although Class I groups are labelled more readily than are Class II groups they are only slightly more susceptible to reduction by ascorbate than the Class II groups, indicating no major difference in environment. The spectral difference observed between immobilized and mobile spin-labels with both Class I and Class II groups labelling is not reflected in widely different reduction kinetics for these two spectral components. Solubilization of the enzyme in an active form does not change the protein structure in terms of increased accessibility of the SH-groups to reduction by ascorbate. The results are discussed in terms of the location of the different SH-groups and the origins of the differences in mobility evident in the EPR spectra of the spin-labelled SH-groups.

## Introduction

The Na,K-ATPase is an integral membrane protein, which is responsible for the active transport of $Na^+$ and $K^+$ across the cell membrane [1]. The protein part of the molecule consists of two subunits, $\alpha$ (molecular mass 112 kDa) and $\beta$ (molecular mass 36 kDa), which are present in equimolar amounts. The oligomeric structure of the enzyme in the membrane is probably an $(\alpha\beta)_2$-diprotomer, although this is still a point of debate (see Ref. 2 for a discussion).

The sulphydryl groups of the enzyme have been studied extensively, since covalent modification of these alter or abolish the enzymatic activity in a ligand-dependent manner [3–5]. The SH-groups are thus thought to play an essential role in maintaining an active protein structure, and a vital SH-group in the ATP-binding site has been described in detail [6].

We have previously classified the SH-groups of the shark rectal gland Na,K-ATPase as follows: a number of groups (Class I, approximately two per $\alpha$-subunit) can be reacted with *N*-ethylmaleimide (NEM) in the presence of glycerol or sucrose with no effect on the overall or partial reactions of the enzyme. In addition to these groups, about 3–4 groups per $\alpha$-subunit can be modified with NEM in the absence of glycerol: these are termed Class II groups [7,8]. Modification of these groups leads to loss of Na,K-ATPase activity. One of the Class II-groups can be protected by ATP in the presence of $K^+$ from modification by NEM. In this case, the enzyme retains the ability to phosphorylate from ATP, but the affinity for $K^+$ is drastically reduced, which leads to loss of overall Na,K-ATPase

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Abbreviations: $\alpha$, the 112 kDa catalytic subunit of the Na,K-ATPase; $\beta$, the 36 kDa glycosylated subunit of the Na,K-ATPase; CDTA, *trans*-1,2-cyclohexylenedinitrilotetraacetic acid; DMF, dimethylformamide; EPR, electron paramagnetic resonance; SH-groups, sulphydryl groups; NEM, *N*-ethylmaleimide; $C_{12}E_8$, octaethyleneglycol dodecyl monoether; 5-MSL, 3-maleimido-1-oxyl-2,2,5,5-tetramethylpyrrolidine; 6-MSL, 4-maleimido-1-oxyl-2,2,6,6-tetramethylpiperidine; 5-MMSL, 3-(maleimidomethyl)-1-oxyl-2,2,5,5-tetramethylpyrrolidine; 5-MeMMSL, *trans*-3-methoxycarbonyl-4-(maleimidomethyl)-1-oxyl-2,2,5,5-tetramethylpyrrolidine; 5-HxMMSL, *trans*-3-hexoxycarbonyl-4-(maleimidomethyl)-1-oxyl-2,2,5,5-tetramethylpyrrolidine; 5-DMAMMSL, *trans*-3-(2'-dimethylaminoethoxy)carbonyl-4-(maleimidomethyl)-1-oxyl-2,2,5,5-tetramethylpyrrolidine; 5-TMAMMSL, *trans*-3-(2'-trimethylaminoethoxy)carbonyl-4-maleimidomethyl-1-oxyl-2,2,5,5-tetramethylpyrrolidine.

activity [9–11]. The kinetics for NEM-inactivation of shark rectal gland Na,K-ATPase are rather complex, and have previously been dealt with in detail [12].

The purpose of the present paper is to investigate and compare the properties of the Class I and II groups, in order to explain the differences between the two sets of sulphydryl groups and to obtain structural information about the enzyme. To this end, we use a number of different maleimide-nitroxide derivatives (see Fig. 1), and study the inactivating properties, incorporation into the enzyme and location of the spin-labels when attached to the protein.

The location of the SH-groups within the enzyme-protein can be probed by the accessibility of the nitroxide moiety to reduction by, for example, ascorbate. Thereby a possible discrimination between SH-groups giving rise to mobile and motionally restricted EPR-signals can be attempted, and the difference between Class I and Class II groups can also be investigated. Differential reduction rates of spin-labels located at different depths in the membrane have been demonstrated previously for spin-labelled lipids in bilayer model membranes [13]. An additional aspect is that a homogeneous population of SH-groups should follow simple reduction kinetics, whereas heterogeneity could give rise, for example, to multi-phasic reduction of the EPR-signal.

## Materials and Methods

### *Spin-label preparation*

The spin-labels used are shown in Fig. 1 and were obtained from Aldrich (in the case of 5-MSL, 5-MMSL and 6-MSL) or synthesized according to Ref. 14 (5-MeMMSL) or by similar methods which will be published elsewhere (5-HxMMSL, 5-DMAMMSL and 5-TMAMMSL).

### *Enzyme preparations and labelling*

Na,K-ATPase from the rectal gland of *Squalus acanthias* was prepared as described previously [15], but omitting the treatment with saponin. The Na,K-ATPase constituted typically 70% of the total protein (determined as the content of $\alpha$- and $\beta$-subunits from SDS gel electrophoresis), and the specific activity was approx. 1500 $\mu$mol ATP hydrolysed/mg protein per h. Na,K-ATPase activity and protein content were determined as previously described [16]. The membrane fragments produced by this preparation are non-vesicular, i.e., both membrane faces are exposed to the same aqueous phase.

### *Maleimide-labelling of Class I groups*

Class I-SH groups in the Na,K-ATPase membranes were spin-labelled under conditions where the Na,K-ATPase activity is unaffected, by 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), with 0.02–0.1 mM spin-label (concentration depending on the reactivity of the label) for 60 min at 23°C. 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*. 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 EPR measurements. This was done essentially as described earlier [17,18]. Spin-labelled membranes were solubilized with $C_{12}E_8$ at a detergent/protein ratio of 1:1 (w/w), whereby only the Na,K-ATPase is solubilized [17]. The solubilized protein was precipitated from the supernatant 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

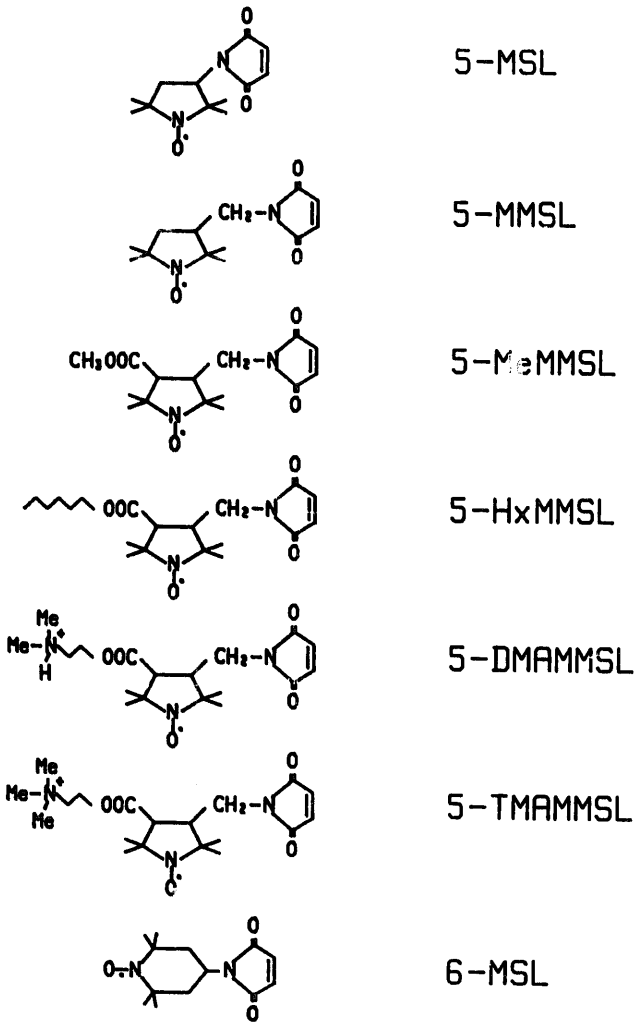



Fig. 1. Structures of nitroxide-derivatives bearing the maleimide group.

lipids only, were collected by centrifugation, washed and used for the EPR experiments.

*Maleimide-labelling of Class II groups*

Prelabelling of Na,K-ATPase with NEM to block Class I SH-groups and sulphydryl groups of non-Na,K-ATPase proteins in the membrane preparations was performed as follows (see Ref. 8 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 200000 × *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 [7]: 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 centrifugation in 20 mM histidine (pH 7.0 at 20°C) and 25% (v/v) glycerol at 200000 × *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 (before washing) was less than 1%. These concentrations of organic solvent in the incubation medium had no effect on enzyme activity.

*EPR spectroscopy*

Samples for EPR spectroscopy were prepared according to the following protocol [19]: 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 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. EPR spectra were recorded on a Varian E-3 Century Line 9 GHz spectrometer equipped with nitrogen gas flow temperature regulation and interfaced to an IBM/PS2 computer with a RTI 815 interface (Analog Devices). Conventional, in-phase, absorption EPR spectra were recorded with a modulation frequency of 100 kHz and a modulation amplitude of 1.6 G peak-to-peak. Further details of the EPR spectroscopy are given in Ref. 19. Graphical presentation of EPR-spectra was performed using a programme developed by L.I. Horváth.

*Reduction of spin-labels with ascorbate*

Reduction of the EPR-signal by ascorbate was followed at 22°C or 3°C, and the protocol was as follows: the labelled enzyme was pelleted in 30 mM histidine (pH 7.4 at 37°C)/100 mM NaCl/1 mM CDTA, and resuspended and homogenized in a small volume of the same buffer. Ascorbate (adjusted to pH 7.4) was added as a concentrated solution, and the sample was immediately transferred to the EPR-cavity for measuring. The time elapsed from addition of ascorbate to start of data collection was typically 60 to 90 s. Reduction of the EPR-signal was recorded by tuning to a given spectral position and following the decay of signal intensity with time.

*Incorporation of spin-label into the protein*

Binding of the maleimide spin-label to the protein was determined by monitoring the decrease in the EPR-signal from the free aqueous spin-label. The spectrometer was tuned to the low-field line, and the reduction in signal intensity was followed with time at 37°C. Parallel measurements of inactivation of the Na,K-ATPase were performed as described above. Control experiments, either with maleimide spin-label (5-MSL), which had been blocked in the maleimide functional group by reaction with amine or with enzyme fully labelled with NEM, showed that there was no rapid reduction in lineheight of the free spin label when it was unable to bind covalently to the protein.

*Preparation of $C_{12}E_8$-solubilized Na,K-ATPase*

Solubilization in $C_{12}E_8$ was performed as follows: to a membraneous suspension in buffer (20 mM histidine, 1 mM CDTA, 100 mM NaCl pH 7.4) was added one-fourth volume $C_{12}E_8$ in the same buffer at 4°C, with a detergent/protein ratio of 2:1 (w/w). The suspension was centrifuged for 45 min in a Beckman Airfuge at full speed, and the clear supernatant used for EPR-experiments.

*Data analysis*

Fitting of the time-dependence of inactivation curves to a sum of exponentials was performed using the programme GraphPAD Inplot version 3.00 (GraphPAD Software, San Diego, CA).

## Results

*Kinetics of inactivation of Na,K-ATPase by maleimide nitroxides*

Na,K-ATPase Class II SH-groups were spin-labelled at 37°C after the Class I SH-groups had been prelabelled with NEM. Labelling of Class II SH-groups at

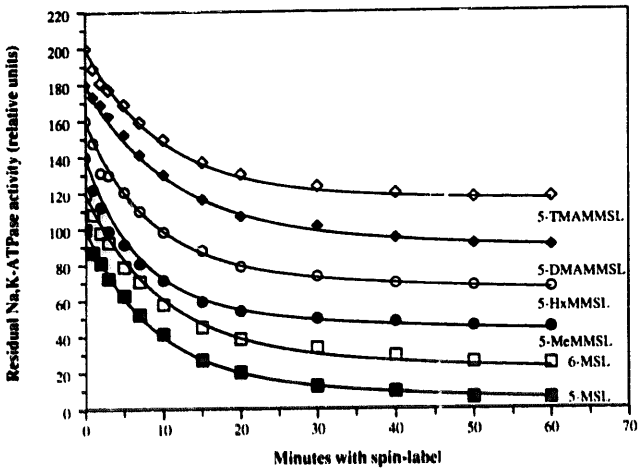



Fig. 2. Time dependence of inactivation of Na,K-ATPase by different maleimide nitroxide-derivatives. Reaction of shark Na,K-ATPase with the maleimide derivatives was performed at 37°C in the presence of 150 mM KCl, 3 mM ATP and 5 mM CDTA in histidine buffer at pH 7.4. The protein concentration was 0.8 mg/ml and the final maleimide concentrations, [SL], were: 0.1 mM (5-MSL, filled squares; and 6-MSL, open squares), 0.2 mM (5-MeMMSL, filled circles), 0.03 mM (5-HxMMSL, open circles) and 0.04 mM (5-DMAMMSL, filled diamonds, and 5-TMAMMSL, open diamonds). At the indicated time-points the residual Na,K-ATPase activity was measured as described in Methods. The curves are displaced vertically (in steps of 20 units) to ease comparison, the total activity being 100 units for all six samples. Bi-exponential curve fits with the parameters given in Table I are shown.

37°C in the presence of 150 mM $K^+$ and 3 mM ATP yields inactivation of overall Na,K-ATPase activity, but the ability to phosphorylate the enzyme is retained [11].

The time courses of inactivation of the Na,K-ATPase on reaction with six of the seven different maleimide spin-labelled derivatives are given in Fig. 2 (note that the curves have been displaced vertically for ease of comparison). The spin-label concentrations used in the experiments shown in Fig. 2 were such that the rates of inactivation lie in the same range for all the spin-labels. For all seven labels the inactivation is biphasic. The inactivation curves can be resolved into two exponentials [12], one with an amplitude of about 82% of the activity which is lost rapidly, and the remaining 18% having a 6–10-fold lower rate of disappearance. The observed rate-constants ($k_{1,obs}$ and $k_{2,obs}$) obtained from such a bi-exponential fit of the data in Fig. 2 are given in Table I. The effective second-order rate constants for inactivation (calculated using the spin-label concentrations given in Table I) vary by a factor of up to 5–6 between the different labels. The more hydrophobic hexyl ester (5-HxMMSL) is the most effective, whereas the charged derivatives (5-DMAMMSL and 5-TMAMMSL) have inactivation rates comparable to that of the parent underivatized spin-label (5-MMSL). The methyl ester (5-MeMMSL) inactivates more slowly than does 5-MMSL, as also do the labels with a shorter maleimide-nitroxide linkage (5-MSL and 6-MSL), in the major inactivation phase.

It should be noted that the kinetics for inactivation of shark rectal gland Na,K-ATPase by maleimide are rather complex [12].

*EPR-spectra and label incorporation*

The EPR-spectra recorded at 22°C of Na,K-ATPase labelled on Class II groups with the seven different maleimide-nitroxide derivatives are given in Fig. 3. Labelling was carried out using the spin-label concentrations given in Table I. The major portion of the spin-label is strongly immobilized on the conventional EPR time scale for all seven spectra. The presence of the sharp mobile spectral component does not correlate with the rates of inactivation in either the fast or the slow phases. For 5-HxMMSL, which shows the most rapid inactivation rates, the mobile component is almost completely absent. The lower-most spectrum in Fig. 3 shows $C_{12}E_8$-solubilized Na,K-ATPase labelled with 5-MSL.

Inactivation of Na,K-ATPase activity is related to incorporation of spin-label into the protein, as shown in Fig. 4. Here the decrease in the height of the low-field EPR-line of the free spin-label (at approx. 3270 G) is followed with time. Within the first 10–15 min after addition of spin-label to the enzyme at 37°C there is a marked decrease in the line-height of the free spin-label, see Fig. 4. This is due to reaction of the spin-label with the protein, which leads to much broader (and thus weaker) lines. The time-course of the decrease in lineheight consists of two components, a relatively rapid component which can be fitted by a single exponential with a rate-constant of 0.38 $min^{-1}$,

TABLE I

*Inactivation of shark rectal gland Na,K-ATPase (Class II) by spin-labelled maleimide derivatives at 37°C*

The data shown in Fig. 2 are fitted by a sum of two exponentials of the form:

$$A(t) = 82 \cdot \exp(-k_{obs,1} \cdot t) + 18 \cdot \exp(-k_{obs,2} \cdot t)$$

where $k_{obs,1}$ and $k_{obs,2}$ are variables. The estimated standard errors of the values obtained for the rate constants are given in percent.

| Label (concn.) | $k_{obs,1}$ ($min^{-1}$) | $k_{obs,2}$ ($min^{-1}$) |
|---|---|---|
| 5-MSL (100 μM) | 0.12 (2.6%) | 0.019 (11.4%) |
| 6-MSL (100 μM) | 0.13 (2.3%) | 0.019 (10%) |
| 5-MMSL [a] (30 μM) | 0.11 (4.2%) | 0.0095 (23%) |
| 5-MeMMSL (200 μM) | 0.18 (4.4%) | 0.020 (18%) |
| 5-HxMMSL (30 μM) | 0.14 (5.1%) | 0.015 (24%) |
| 5-DMAMMSL (40 μM) | 0.086 (2.8%) | 0.011 (16%) |
| 5-TMAMMSL (40 μM) | 0.099 (3.1%) | < 0.001 |
| NEM [a] (100 μM) | 0.088 (5.5%) | 0.0074 (40%) |

[a] Data not shown in Fig. 2.

and a slow, almost linear decrease. The magnitude of the rapid component corresponds to about 9 $\mu$M free spin-label removed from solution, i.e., to incorporation of about four spin-labels per phosphorylation site. The slow disappearance of free spin-label corresponds to a labelling of an additional 2–3 moles of SH-groups per mole phosphorylation site after 20 min of incubation.

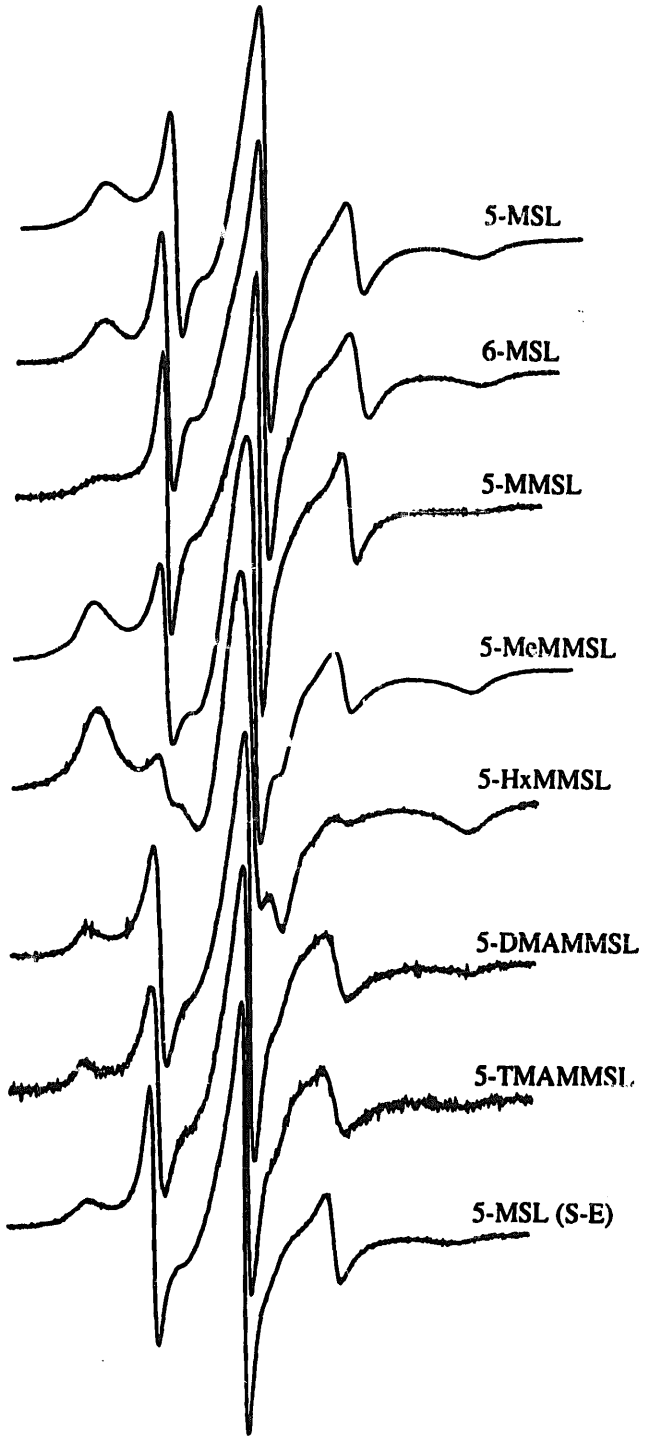



Fig. 3. EPR spectra recorded at 22°C of Na,K-ATPase spin-labelled at Class II groups with different maleimide nitroxide derivatives. The lower-most spectrum represents 5-MSL-labelled supernatant enzyme, solubilized with $C_{12}E_8$. Total scan width = 100 Gauss.

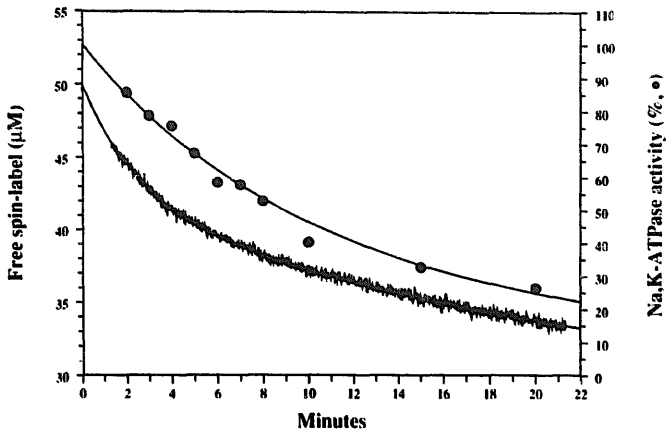



Fig. 4. Comparison between incorporation of spin-label and loss of Na,K-ATPase activity. NEM-prelabelled Na,K-ATPase was incubated with 5-MSL at 37°C, and the loss of Na,K-ATPase activity (filled circles) and the decrease in the height of the low-field EPR-line of the free aqueous 5-MSL were followed with time. The inactivation data are fitted by the expression $Y(t) = 82 \cdot \exp(-0.097 \cdot t) + 18$, and the decrease in the EPR-line height (converted to units of free spin-label concentration in $\mu$M) is fitted by the expression $Y(t) = 8.8 \cdot \exp(-0.38 \cdot t) + 14 \cdot \exp(-0.04 \cdot t) + 27.2$, where $t$ is in min.

A slow decrease of EPR-signal from free spin-label was also observed in a similar experiment with an enzyme preparation, where both Class I and Class II SH-groups had been modified with NEM. It is therefore possible that the slow component is related to other reactions than modification of SH-groups.

The pseudo-first-order rate constant for inactivation (measured in parallel with the spin-label incorporation) is about 0.09 $min^{-1}$ with 50 $\mu$M spin-label. Apparently the spin-label incorporation is faster than the inactivation of the enzyme. This could suggest that the inactivation is due to modification only of a subset of the groups that are spin-labelled.

*Reduction of the free spin-labels by ascorbate*

In the remaining part of the paper the kinetics of ascorbate-reduction of the various spin-labels attached at Class I or Class II-groups is studied in an attempt to characterize the environment (i.e., location) of the labelled SH-groups.

Fig. 5 shows, in a semilogarithmic plot, the reduction by ascorbate of six different free spin-labels in buffer at 1°C. The height of the low-field line is followed in time with the ascorbate concentrations given in Table II. The curves shown in Fig. 5 are not straight lines, but tend to curve upwards. This indicates that the reduction process is not a simple bimolecular reaction between the spin-label and (excess) ascorbate. The reduction curve for 6-MSL is almost linear, a bi-exponential fit shows that only about 5% of the spin-intensity is reduced at a slow rate. Reduction rates and second order rate constants deduced from the major component are given in Table II for all seven spin-

labels. The rate of reduction is proportional to the ascorbate concentration and the mean values of the second-order rate constant obtained at different concentrations are given in the table. Rate constants are 2–5-fold larger at 22°C than at 1°C. Note the large difference in the second-order rate constant for reduction between the spin-labels with 5-membered nitroxide rings (2 to 40 $M^{-1}$/min at 1°C) and the six-membered ring spin-label 6-MSL (about 240 $M^{-1}$/min at 1°C).

*Kinetics of reduction of protein-bound 5-MSL by ascorbate at 22°C*

The kinetics of reduction of 5-MSL bound to Class II groups was investigated by following the time- and concentration dependence of the intensity of the low-field line from immobilized spin-labels upon addition of ascorbate (see Fig. 6). About 70% of the line-height disappears within 20 min at the highest ascorbate concentration used (36 mM), with the remaining disappearing slowly. The decays shown in Fig. 6 are fitted by

TABLE II

*Rates of reduction of free maleimide spin-label derivatives in aqueous solution using ascorbate as reducing agent*

Rates of reduction were deduced from a single-exponential fit to data such as shown in Fig. 5, allowing for a minor residual component, which is reduced much more slowly than the major signal. The observed pseudo-first-order rate constants ($min^{-1}$) are converted to second-order rate constants ($M^{-1}$/min) for each spin-label.

| Label | Temp. (°C) | [Ascorbate] (mM) | Reduction rate ($min^{-1}$) | Rate constant ($M^{-1}$/min) | Rate constant average ($M^{-1}$/min) |
|---|---|---|---|---|---|
| 5-MSL | 1 | 10 | 0.058 | 5.8 | 5.5 |
| | 3 | 30 | 0.16 | 5.2 | |
| | 22 | 10 | 0.19 | 19 | 22 |
| | 22 | 10 | 0.25 | 25 | |
| | 22 | 10 | 0.21 | 21 | |
| 6-MSL | 1 | 0.5 | 0.12 | 230 | 240 |
| | 3 | 0.5 | 0.12 | 240 | |
| | 22 | 0.5 | 0.35 | 700 | 570 |
| | 22 | 0.5 | 0.23 | 460 | |
| | 22 | 0.5 | 0.27 | 540 | |
| 5-MMSL | 3 | 30 | 0.054 | 1.8 | 1.8 |
| | 3 | 30 | 0.056 | 1.9 | |
| | 3 | 50 | 0.086 | 1.7 | |
| | 22 | 10 | 0.078 | 7.8 | 8.2 |
| | 22 | 10 | 0.092 | 9.2 | |
| | 22 | 30 | 0.23 | 7.6 | |
| 5-MeMMSL | 1 | 10 | 0.13 | 13 | 13 |
| | 3 | 15 | 0.18 | 12 | |
| | 22 | 5 | 0.23 | 46 | 43 |
| | 22 | 5 | 0.20 | 40 | |
| 5-HxMMSL | 1 | 10 | 0.17 | 17 | 14 |
| | 3 | 10 | 0.12 | 12 | |
| | 22 | 5 | 0.33 | 66 | 66 |
| | 22 | 5 | 0.33 | 66 | |
| 5-DMAMMSL | 1 | 2 | 0.073 | 36 | 33 |
| | 3 | 5 | 0.15 | 30 | |
| | 22 | 2 | 0.13 | 66 | 69 |
| | 22 | 5 | 0.36 | 72 | |
| 5-TMAMMSL | 1 | 2 | 0.089 | 45 | 41 |
| | 3 | 5 | 0.18 | 36 | |
| | 22 | 2 | 0.16 | 80 | 90 |
| | 22 | 5 | 0.50 | 100 | |

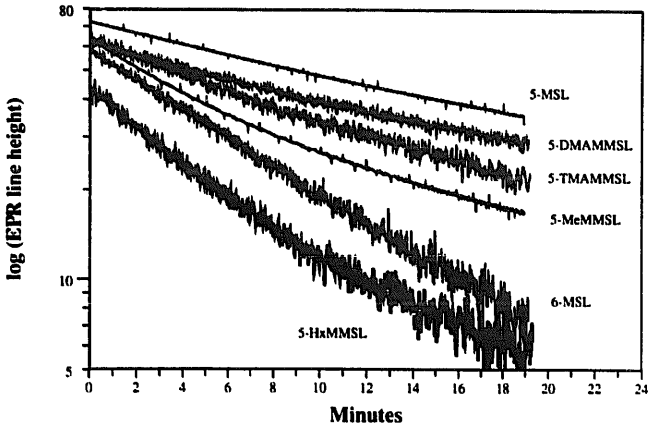



Fig. 5. Reduction of free spin-labels in aqueous solution at 1°C using ascorbate as reductant. The time dependence of the decrease in height of the low-field line of the EPR spectrum is shown on a log-scale for six different maleimide spin-label derivatives in the presence of ascorbate. The concentrations of ascorbate used for the six different experiments are given in Table II.

single exponentials with decay-constants of 0.15 $min^{-1}$ (with 9.1 mM ascorbate, upper trace), 0.22 $min^{-1}$ (with 18.2 mM ascorbate, middle trace) and 0.35 $min^{-1}$ (with 36.4 mM ascorbate, lower trace). From these data a mean second-order rate constant of $0.013 \pm 0.004$ $mM^{-1}/min$ can be estimated. A bi-exponential fit aimed at determining the rate-constants for the slowly reduced spin-labels gives the result that the slow phase is at least 15–20-times slower than the rapid phase. Whether this bi-phasic reduction is due to heterogeneity of the spin-labels bound to the enzyme or to the intrinsic bi-phasic behaviour of the reduction of the spin-labels (cf. Fig. 5) is uncertain. The results shown in Fig. 6, however, indicate that the major portion of the spin-label can be reduced by ascorbate, and that

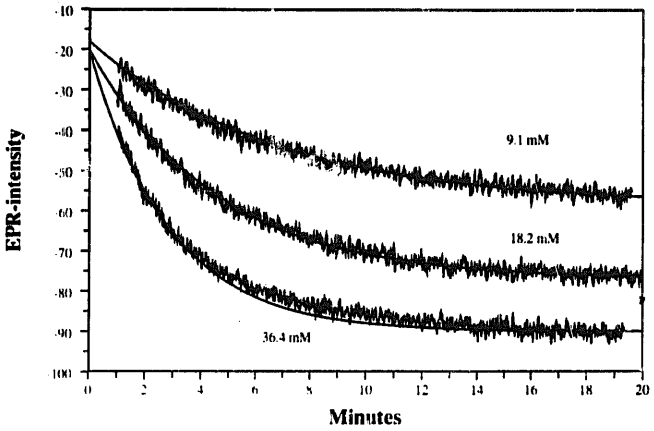



Fig. 6. Kinetics of reduction by ascorbate of 5-MSL bound to Class II groups. The intensity of the low-field line corresponding to the immobilized 5-MSL spin-labels was followed with time, and the decays are fitted by single exponentials with decay-constants of 0.15 $min^{-1}$ (with 9.1 mM ascorbate, upper trace), 0.22 $min^{-1}$ (with 18.2 mM ascorbate, middle trace) and 0.35 $min^{-1}$ (with 36.4 mM ascorbate, lower trace).

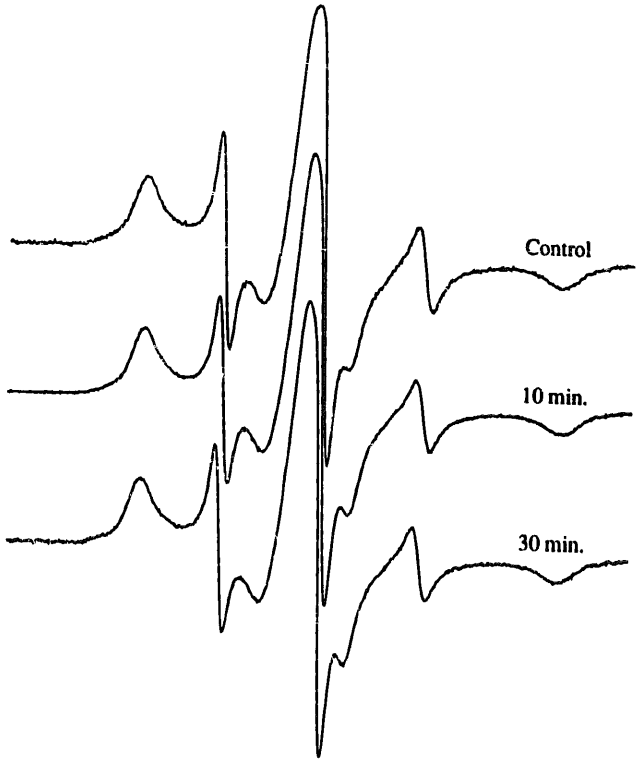



Fig. 7. Comparison of the EPR spectra lineshapes at 4°C of 5-MSL spin-labelled Na,K-ATPase after various times of reduction with ascorbate. Labelled membranes were incubated with 9.1 mM ascorbate for 0 (upper spectrum), 10 (middle spectrum) or 30 min (lower spectrum) at 22°C. After incubation the enzyme was diluted 50-fold into histidine-glycerol buffer at 0°C and washed twice by centrifugation. Total scan width = 100 Gauss. Spectra were normalized to the same central lineheight.

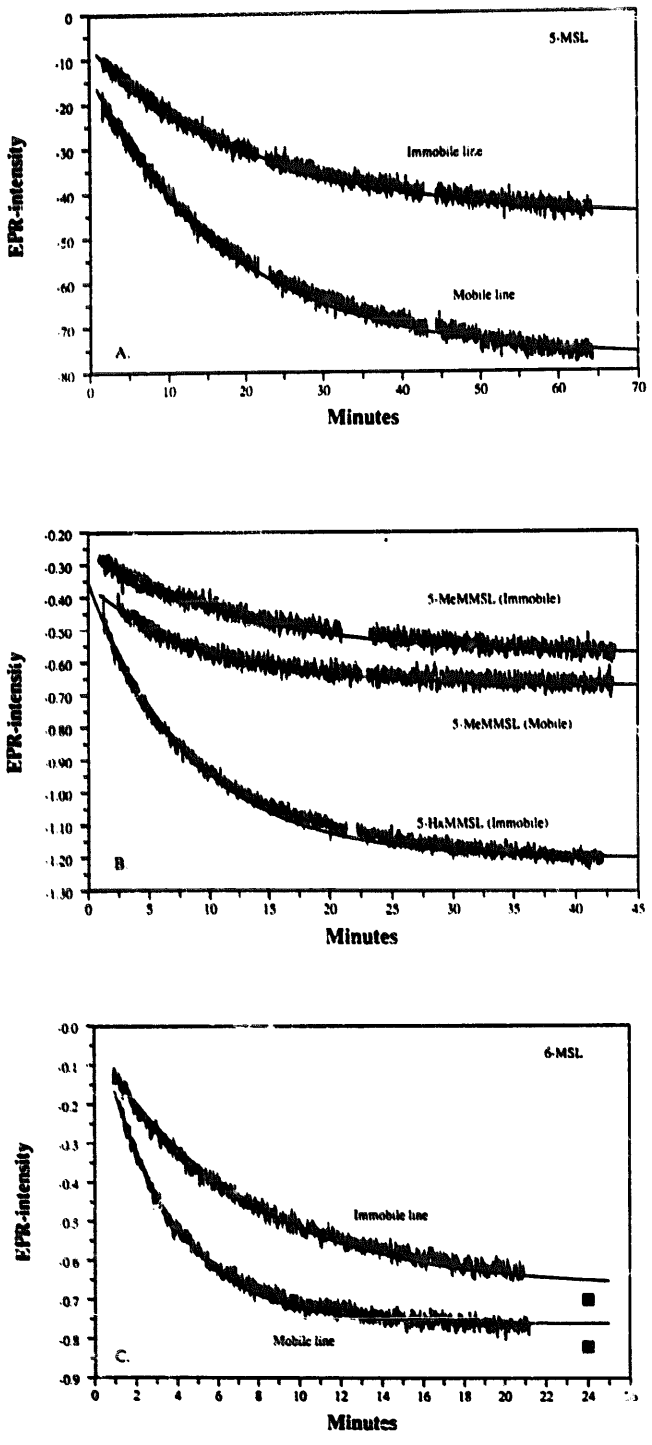
the rate of reduction increases progressively with the ascorbate concentration.

Fig. 7 shows that reduction for either 10 or 30 min with 9.1 mM ascorbate at 22°C does not change the shape of the EPR-spectra from 5-MSL labelled Class II groups appreciably. This result suggests that rates of reduction of the mobile and immobile spectral components are similar, and that there is no large-scale heterogeneity within these two components.

*Reduction of spin-labelled Class II-groups by ascorbate*

Reduction of the EPR-signal by ascorbate at 3°C and at 22°C was followed in time by monitoring the lineheight of the mobile component or of the motionally restricted component (in separate experiments). Fig. 8 shows the results obtained at 3°C with 5-MSL (panel A), 5-MeMMSL and 5-HxMMSL (the methyl and hexyl esters, panel B) and the 6-MSL label (panel C). For 5-MSL, approx. 80% of the EPR-signal is reduced with kinetics approaching a single exponential for both the motionally restricted and the mobile spin-labels. The rate of reduction is slightly more rapid for the mobile spin-labels than for the motionally restricted labels (cf. Table III below). For 5-MeMMSL a

comparable reduction rate is obtained at a considerably lower ascorbate concentration than for 5-MSL, whereas reduction of 5-HxMMSL is more rapid than the reduction of 5-MSL at the same ascorbate concentration. For 6-MSL the reduction is almost single-exponential for both the mobile and the immobile groups, the value for the base-line level is shown by the squares in Fig. 8C. The residual, slowly reduced component thus comprises less than 10% of the total signal (Fig. 8C).




For all seven spin-labels used, the rate-constants for reduction at 3°C are given in Table III for both mobile and immobilized spin-labels, calculated as second-order rate constants (in units of $M^{-1}$/min). Note that the mobile component is reduced at a somewhat higher rate than the motionally restricted spin-label in most cases. When normalized relative to the reduction rate-constant for the corresponding free spin-label (cf. Table II), the reduction rate of the hexyl ester derivative (5-HxMMSL) is considerably smaller than that of the parent compound 5-MMSL, and those of the positively charged amine derivatives (5-DMAMMSL and 5-TMAMMSL) as well as that of 5-MeMMSL have about the same rate as 5-MMSL. The difference in reduction rates between the tertiary (5-DMAMMSL) and quaternary (5-TMAMMSL) amine derivatives may be due to a shift in $pK_a$ of the former, resulting in it being only partially charged when bound to the protein. The normalized (and also absolute) reduction rates of all the methylmaleimide derivatives (except 5-HxMMSL) at 3°C are appreciably faster than that of 5-MSL, presumably due to steric hindrance in 5-MSL of motion of the nitroxide relative to the protein.

The results for the reduction at 22°C of the 6-MSL, 5-MSL, 5-MMSL and 5-HxMMSL derivatives are shown also in Table III. Here the same pattern is observed as at low temperature, with the mobile spin-labels being reduced more rapidly than the immobilized ones. Interestingly, the rates of reduction relative to those of the free spin-label are higher at 22°C than at 3°C for the 5-MSL and 6-MSL labels, whereas for the 5-MMSL and 5-HxMMSL labels the relative reduction rates are comparable at the two temperatures.

The effect of solubilization of the enzyme in $C_{12}E_8$ was also investigated. Fig. 3 (lower panel) shows the spectrum of 5-MSL labelled on Class II groups after

Fig. 8. Reduction at 3°C of spin-labelled Class II-groups by ascorbate. (A) The intensity of the low-field line corresponding to the immobilized 5-MSL spin-labels (upper trace) was followed with time, and the decay is fitted by a single exponential with a decay-constant of 0.046 $min^{-1}$. The intensity of the low-field line corresponding to the mobile spin-labels (lower trace) was followed with time, and the decay is fitted by a single exponential with a decay-constant of 0.055 $min^{-1}$. (B) Reduction of 5-MeMMSL and 5-HxMMSL. The intensities of the low-field lines of 5-MeMMSL were followed with time, and the decays are fitted by single exponentials with decay-constants of 0.08 and 0.105 $min^{-1}$, for the immobilized spin-labels (upper trace) and the mobile spin-label (middle trace), respectively. The intensity of the low-field line corresponding to the immobilized 5-HxMMSL spin-labels was followed with time, and the decay is fitted by a single exponential with a rate-constant of 0.12 $min^{-1}$ (lower-most trace). (C) The intensities of the low-field lines of 6-MSL were followed with time, and the decays are fitted by single exponentials with decay-constants of 0.14 and 0.30 $min^{-1}$, for the immobilized spin-labels (upper trace) and the mobile spin-label (lower trace), respectively. The baseline levels are also shown for the traces (filled squares).

solubilization in $C_{12}E_8$ (supernatant enzyme is used to ensure that we are monitoring truly solubilized enzyme). The main effect of solubilization is a sharpening of the mobile component in the EPR-spectrum. The decay curves resulting from reduction of the EPR-signal by ascorbate for both membranous and solubilized enzymes were fitted to a sum of two exponentials with the constraint that the EPR-signal must decay to zero at infinite time. The faster kinetic component was found to account for approx. 75% of the total spin-label intensity, for both mobile and motionally restricted spin-labelled groups. A somewhat higher reduction rate of the more rapid component is observed for mobile spin-labels relative to immobilized ones (approx. 28 $M^{-1}$/min vs. 19 $M^{-1}$/min, for the membranous enzyme), but these rates were not affected appreciably by solubilization. The slow-phase reduction is 10–15-times slower than the rapid phase, both for solubilized and membrane-bound enzyme. Reduction rates obtained from single-exponential fits for the solubilized enzyme is included for comparison in Table III.

TABLE III

*Rates of reduction of maleimide spin-label derivatives bound to Class II SH-groups using ascorbate as reducing agent*

Rates of reduction were estimated from single-exponential fits such as those shown in Fig. 8, and converted to second-order rate constants for each spin-label.

| Label | Temp. (°C) | [Ascorbate] (mM) | Reduction rate ($min^{-1}$) | Rate constant | |
|---|---|---|---|---|---|
| | | | | Abs. [a] | Rel. [b] |
| 5-MSL | 3 | 45.5 | imm. [c] 0.046 | 1.0 | 0.18 |
| | 3 | 45.5 | mob. [c] 0.055 | 1.2 | 0.22 |
| | 22 | 9.1 | imm. 0.13 | 14 | 0.64 |
| | 22 | 18.2 | imm. 0.27 | 15 | 0.68 |
| | 22 | 9.1 | mob. 0.21 | 23 | 1.1 |
| | 22 | 18.2 | mob. 0.37 | 20 | 0.95 |
| 6-MSL | 3 | 1.8 | imm. 0.14 | 78 | 0.33 |
| | 3 | 1.8 | mob. 0.30 | 170 | 0.69 |
| | 22 | 0.46 | imm. 0.13 | 280 | 0.49 |
| | 22 | 0.46 | mob. 0.17 | 380 | 0.67 |
| 5-MMSL | 3 | 45.5 | imm. 0.095 | 2.1 | 1.2 |
| | 3 | 45.5 | mob. 0.14 | 3.0 | 1.7 |
| | 22 | 9.1 | imm. 0.082 | 9.0 | 1.1 |
| | 22 | 22.7 | imm. 0.16 | 7.0 | 0.85 |
| | 22 | 9.1 | mob. 0.12 | 13 | 1.6 |
| | 22 | 22.7 | mob. 0.20 | 8.9 | 1.1 |
| 5-MeMMSL | 3 | 9.1 | imm. 0.08 | 8.8 | 0.70 |
| | 3 | 9.1 | mob. 0.11 | 12 | 0.91 |
| 5-HxMMSL | 3 | 45.5 | imm. 0.13 | 2.8 | 0.20 |
| | 22 | 9.1 | imm. 0.14 | 15 | 0.23 |
| 5-DMAMMSL | 3 | 4.45 | imm. 0.090 | 20 | 0.60 |
| | 3 | 4.45 | mob. 0.10 | 22 | 0.67 |
| 5-TMAMMSL | 3 | 1.8 | imm. 0.080 | 44 | 1.1 |
| | 3 | 1.8 | mob. 0.080 | 44 | 1.1 |
| 5-MSL [d] | 22 | 18.2 | imm. 0.30 | 16 | 0.77 |
| | 22 | 18.2 | mob. 0.34 | 18 | 0.86 |

[a] Second-order rate constant ($M^{-1}$/min).
[b] Relative to reduction of free spin-label at the same temperature.
[c] imm., refers to the spectral component corresponding to the immobile spin-labels and mob. to the mobile spin-labels.
[d] $C_{12}E_8$ solubilized supernatant enzyme.

TABLE IV

*Rates of reduction of maleimide spin-label derivatives bound to Class I SH-groups using ascorbate as reducing agent*

Rates of reduction were estimated as described in Table III.

| Label | Temp. (°C) | [Ascorbate] (mM) | Reduction rate ($min^{-1}$) | Rate constant | |
|---|---|---|---|---|---|
| | | | | Abs. [a] | Rel. [b] |
| 5-MSL | 22 | 9.1 | imm. [c] 0.24 | 26 | 1.2 |
| | 22 | 9.1 | mob. [c] 0.28 | 30 | 1.4 |
| 5-HxMMSL | 3 | 36.4 | imm. 0.13 | 3.4 | 0.24 |
| | 22 | 9.1 | imm. 0.13 | 14 | 0.22 |
| | 22 | 9.1 | mob. 0.19 | 21 | 0.31 |

[a] Second-order rate constant ($M^{-1}$/min).
[b] Relative to reduction of free spin-label at the same temperature.
[c] imm. refers to the spectral component corresponding to the immobile spin-labels and mob. to the mobile spin-labels.

*Reduction of spin-labelled Class I groups*

The reduction kinetics of Na,K-ATPase spin-labelled at Class I groups were followed as for Class II groups, and Table IV shows the rate-constants deduced from the single-exponential fits of the decay curves. There is a minor difference in the rate of reduction between mobile and motionally restricted spin-labels. When normalized with respect to the intrinsic reduction rates of the free spin-labels, the reduction rate of 5-MSL is much faster than that of the hexyl ester derivative (5-HxMMSL). Comparison of the data in Tables III and IV shows that Class I groups labelled with 5-MSL are reduced 1.5–2-fold more rapidly than are Class II groups with the same label, whereas both classes of groups labelled with 5-HxMMSL are reduced at almost the same rate.

## Discussion

The inactivation rates of the Na,K-ATPase by the 5-MSL, 6-MSL and 5-MeMMSL spin-labels are all rather similar to that for NEM, suggesting that the accessibility of the SH-groups essential for activity is similar for all these maleimide derivatives (see Table I). Inactivation by the more hydrophobic 5-HxMMSL spin-label is considerably more rapid than that by NEM, but the charged spin-labels 5-DMAMMSL and 5-TMAMMSL also inactivate the enzyme more rapidly than does NEM as does also the parent compound 5-MMSL. Combining these results, suggests that the

SH-groups necessary for activity are readily accessible from the aqueous phase, but may be located close to the polar-apolar interface of the membrane. The relatively rapid inactivation by the 5-HxMMSL spin-label may be due to its concentration in the membrane by anchoring of the hexyl chain in the lipid phase in such an orientation that the maleimide group may react readily with the essential SH-groups. The rates of inactivation do not correlate directly with the rates of reduction of the spin-labels by ascorbate that are discussed below, but this is probably because only one or a few of the various SH-groups that are labelled are responsible for the inactivation kinetics. It is also found that the rate of inactivation is slower than the rate of incorporation of the 5-MSL spin-label into the enzyme (see Fig. 4), again because SH-groups additional to that essential for activity are also labelled.

The intrinsic rates of reduction by ascorbate determined for the various labels free in aqueous solution are found to differ considerably (see Table II). The reduction rate for 6-MSL free in solution is greater than that for 5-MSL and the other spin-labels containing the 5-membered pyrrolidine ring, because of the inherently greater chemical stability of the latter compared with the 6-membered piperidine ring-containing nitroxides. The reduction rates of the positively charged, ammonium derivatives (5-DMAMMSL and 5-TMAMMSL) in solution are greater than those for 5-MMSL and the other uncharged pyrrolidine nitroxides because of the favourable electrostatic interaction with the negatively charged ascorbate ion. These differences necessitate normalization with respect to the intrinsic rate when comparing the relative reduction rates of the different spin-labels bound to the protein. It should be noted that the normalization is carried out with respect to the rapidly reduced part of the EPR-signal, which is more than 80% of the intensity for the free spin-label and 60–80% for the protein-bound spin-labels.

The normalized rate constant for reduction by ascorbate is slowest for the hydrophobic 5-HxMMSL label bound to Class II SH-groups (Tables III). This is consistent with this spin-label being attached predominantly to SH-groups that are to some extent buried in a hydrophobic environment. At 3°C, the normalized reduction rate of 5-MSL bound to Class II groups is similar to that for 5-HxMMSL, suggesting that 5-MSL reacts preferentially with Class II groups that are also somewhat buried. However, unlike 5-HxMMSL, the normalized reduction rate of 5-MSL is appreciably faster at 22°C, demonstrating that these two labels are not modifying the same Class II groups to identical extents. The 5-MMSL label is bound to some SH-groups that are more accessible to ascorbate in the aqueous phase than are those to which 5-HxMMSL is attached. The positively charged spin-label 5-TMAMMSL has together with 5-MMSL the fastest normalized reduction rates at 3°C of the different labels bound to Class II SH-groups (Table III). This would be consistent with these spin-labels being attached to groups that are more exposed to the aqueous phase than are those modified by 5-HxMMSL. The reduction rates of 5-MeMMSL and 5-DMAMMSL bound to Class II groups are rather comparable which, as in the comparison between 5-TMAMMSL and 5-MMSL, may sugggest that these labels also are attached predominantly to groups accessible to the aqueous phase.

The normalized reduction rate constant for 5-MSL attached to Class I SH-groups is somewhat faster than that of the same label attached to Class II SH-groups, although the difference is not extremely large (cf. Tables III, IV). However, the normalized reduction rate of 5-HxMMSL bound to Class I groups is very similar to that of the same label bound to Class II groups, and in both cases shows very little temperature dependence. This might suggest that the operationally defined distinction between Class I and Class II groups does not correspond to distinct physical locations for the two sets of SH-groups, but rather to subclasses with different reactivities within the same population of SH-groups.

Surprisingly, the mobile and immobile spectral components are reduced with rather similar rates for all the different spin-labels (Tables III and IV). Differences in reduction rates of the two components are mostly less than a factor of two. This is in spite of the fact that the mobile groups clearly have a considerably greater rotational freedom and therefore would be expected to be more accessible to reaction with ascorbate. A possible explanation may be that mobile and immobile components in the spectrum arise from labelling of the same SH-groups, but correspond to different conformations of the enzyme. If the rates of conversion between these conformations were rapid on the timescale of the reduction by ascorbate, the mobile conformation would then provide a pathway by which the entire spin-label population could be reduced and both spectral components would be reduced at approximately the same rate, as is observed. However, the mobile component is nearly absent from the spectra of the 5-HxMMSL spin-label (Fig. 3). Therefore, if the mobile component corresponded to a different conformation for the other labels, this conformation would have to give rise to a strongly immobilized spectrum in the case of the 5-HxMMSL label. A limited conversion between the two spectral components has been observed in response to ligand-induced conformational changes for the enzyme labelled on Class I groups [7], but to a lesser extent than the difference between the degree of incorporation of spin-label intensity into these two components for labelling of Class II groups in the presence of different ligands [8].

Significantly, the rate constants for reduction of the labelled enzyme at 22°C are comparable to those of the free spin-label for nearly all the maleimide-nitroxide derivatives, with the exception of 5-HxMMSL. This suggests that the majority of the SH-groups labelled are rather readily accessible to the aqueous phase. The latter conclusion is consistent with energy transfer experiments between fluorescently labelled ouabain and fluorescently labelled SH-groups which have located the majority of the reactive SH-groups on the intracellular face of the Na,K-ATPase from *Electrophorus electricus* [20].

The primary sequence of the $\alpha$-subunit of the Na,K-ATPase from kidney contains 23 cysteine residues [21], only 6–7 of which are labelled by NEM in the membranous enzyme from *Squalus acanthias* [7] (assuming that the shark and kidney enzyme are largely homologous, cf. Ref. 22). According to recent models for the transmembranous disposition of the Na,K-ATPase [23–25] only 6 of the 23 cysteine residues are contained in the intramembranous section of the protein with the remainder being within the cytoplasmic domain which accounts for most of the mass of the protein. Therefore it is possible that the majority of the residues modified by the maleimide-nitroxides resides within the cytoplasmic domain, which would be consistent with the relatively rapid rates of spin-label reduction by ascorbate. In recent preliminary experiments in which the extramembranous domain of the protein was largely removed by extensive trypsinization as described in Ref. 25, we have found that about one-third of the spin-labels are attached to those fragments of the protein (the 19 kDa C-terminal segment and smaller peptides) that remain associated with the membrane. This corresponds roughly to labelling of 2–3 of the 8 cysteine residues present in these membrane-associated fragments (see Ref. 25).

In summary, the SH-groups essential for activity are most readily labelled by the hydrophobic 5-HxMMSL derivative, which is less readily accessible to reduction by ascorbate, whereas the remainder of the SH-groups labelled by maleimide derivatives appear to be located to the polar surface of the enzyme. The previous interpretation of the distinction between Class I and Class II groups in terms of accessibility from the aqueous phase [7,8] is not supported in detail by the present experiments on the accessibility of the spin-labels to reduction by ascorbate.

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