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AN ELECTRON SPIN PROBE STUDY OF (Na^+ + K^+)-ATPase-CONTAINING MEMBRANES

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Summary

The modulating effect of membrane lipids on enzyme function has been described by several investigators. We have used the spin probe *N*-oxyl-4′,4′-dimethyloxazolidine-12-keto methyl stearate (M 12-NSE) to study this interaction in ox brain membranes enriched with (Na^+ + K^+)-ATPase. This methyl ester of stearic acid is practically insoluble in aqueous media, and consequently spectra of M 12-NSE-labelled preparations are free of "liquid lines".

At least two types of spectra may be obtained when ox brain microsomes are spin labelled with M 12-NSE, indicating the presence of two distinct binding sites. At one site the spin label is relatively unrestricted and gives rise to an isotropic spectrum. A second spectrum, which is obtained from spin label at another site, is similar to that which is observed after incorporation of M 12-NSE into phospholipid bilayers. This suggests that this latter site is within the core of the microsomal membrane.

The two binding sites differ in their affinity for the spin probe. The low affinity site is both more abundant in crude preparations and is more easily removed by detergent treatment; spin labels at this site produce isotropic spectra. The high affinity sites are fewer in number and produce broad spectra. In addition these high affinity sites increase in concentration as the enzyme undergoes purification.

The two sites are quite distinct in their sensitivity to ascorbic acid, the low affinity site showing a considerably greater rate of reduction by this agent.

This study also demonstrates that the delipidation effects of sodium dodecyl sulfate and sodium deoxycholate on (Na^+ + K^+)-ATPase-enriched microsomes from ox brain are not identical.

It is suggested that the two spin probe binding sites represent two different lipid domains, one of which is very closely associated with the (Na^+ + K^+)-

Abbreviations: ESR, electron spin resonance spectroscopy; M 12-NSE, *N*-oxyl-4′,4′-dimethyloxazolidine-12-keto methyl stearate; 5-NS, *N*-oxyl-4′,4′-dimethyloxazolidine-5-keto stearic acid.

ATPase enzyme and may reflect a protein-directed phospholipid specificity for this enzyme.

Introduction

The preparation of ($Na^+ + K^+$)-ATPase containing microsomes by differential centrifugation with or without sodium deoxycholate extraction, can result in products that differ significantly in the specific activity of the enzyme. However, the temperature sensitivity of the enzyme is not altered [1]. In contrast, the binding of ouabain to this enzyme, which is also temperature sensitive, is markedly different after extraction of the microsomal preparations with sodium deoxycholate or other detergents [2]. This temperature sensitivity is best described by the non-linearity found in Arrhenius plots which are believed to arise from phase transitions or phase separations of the membrane lipids [3]. Whether these transitions pertain to a specific lipid or a number of lipids is not clear. The use of biophysical techniques such as fluorescence spectroscopy and ESR to investigate the physical behaviour of lipids is extensively used, and we are currently engaged in an electron spin probe study of microsomal membranes rich in ($Na^+ + K^+$)-ATPase. A number of review articles of this object are now available [4—6]. It is widely believed that both steroid and fatty acid spin probes intercalate in the membrane bilayer [6] and are consequently used to detect physical changes in the characteristics of the lipid matrix. However, it is not yet possible to precisely locate the spin label which may report from specific domains, from the bulk lipid, or from both [7,8]. In addition, the spin probes may also dissolve in the aqueous phase and give rise to "liquid lines" [9, 10]. Spin probes in aqueous solution are rapidly reduced by ascorbic acid and a study of the rate of this reaction can provide information as to the location of the spin probe in the bilayer [11--13]. In the present study we present evidence to show that the spin probe, the *N*-oxyl-4′,4′-dimethyloxazolidine-12-keto derivative of methyl stearate (M 12-NSE) which is essentially insoluble in aqueous media, will bind non-covalently at more than one site to ox brain microsomes rich in ($Na^+ + K^+$)-ATPase. Preliminary communications of this work have been made at recent symposia [27,28].

Materials and Methods

Microsomes and liposomes. The preparation, delipidation procedures, and biochemical assay of the ($Na^+ + K^+$)-ATPase of the ox brain microsomes have been described recently by Charnock et al. [2].

Liposomes were made from dimyristoyl phosphatidylcholine (Serdary Research Laboratories, Ontario) or from the lipids extracted from ox brain microsomes. Weighed amounts of lipid were added to a buffered sucrose solution (0.25 M sucrose, 5 mM Na_2 EDTA, pH 7.6, with Tris) and the mixtures sonicated in a Cole-Parmer Ultrasonic Cleaner, Model 884S-4, until a clear solution was obtained.

Electron spin resonance. The spin label, a methyl ester of stearic acid with the nitroxyl on the 12-C position M (12-NSE), was a grift from Dr. J.K. Raison,

Macquarie University, Sydney, Australia. A methanol solution of M 12-NSE was evaporated in a stream of nitrogen and the suspension of microsomes or liposomes was added to the label. After incubation for 10 min at 37°C, the sample was stored at 0°C until it was used for spectral investigation. There was no difference in the spectra that were obtained whether the labelling procedure was carried out directly in the glass capillary sample tube (1.1 mm internal diameter) or in a separate vessel and subsequently transferred. No sample was stored for longer than 24 h.

The ESR spectra were obtained using a Varian V-4502 EST spectrometer. This instrument is equipped with an Alpha Model 3039 digital NMR gaussmeter for magnetic field calibration and a Varian-4557 temperature controller. The temperature of the sample chamber was examined by a copper-constantin thermocouple, to 0.1°C. Microwave frequencies were monitored with a HPX 532B frequency meter.

A number of methods are currently available for the quantitation and semiquantitation of ESR spectra. Using some approximations, McConnell [14], Kivelson [15] and Freed and Fraenkel [16] have developed mathematical methods for the calculation of rotational correlation times of spin probes incorporated into rigid matrices. These approaches are only applicable to rapid motion, such as that seen as isotropic spectra from spin probes in solution. The ESR spectra of fatty acid spin probes in lipid bilayers are often indicative of anisotropic motion [17,18] and a measure of the membrane fluidity, the order parameter, can be obtained for spectra such as these [17,18]. Other methods that have also been used for broad spectra, make use of one or more of the peak heights and although they are semiquantitative, these simple approaches can be very useful in the interpretation of ESR spectra obtained from spin-labelled biomembranes. In this report we have generally confined our results to a qualitative description of the different spectra; where quantitative data is presented, peak heights are used as described in the legends.

The reduction of M 12-NSE by ascorbic acid was examined by exposing the labelled material to 2 mM ascorbic acid, freshly prepared, (pH 7.5 with Tris) and monitoring the changes in peak heights with time.

Results

In the application of electron spin resonance spectroscopy to the examination of biomembranes, two complications are commonly encountered. One of these is the appearance of "liquid lines" due to spin probe that has dissolved in the aqueous phase [9,10], and a second problem is the possibility of line broadening due to interactions between the electrons of the spin probe molecules when pooling or clustering occurs [19,20]. Both these effects can be avoided however, by reducing the amount of spin probe that is employed, but some limitations must first be defined. Preliminary experiments [27,28] demonstrated that any spectral contribution from the empty capillary tube, the residual spin label on the walls of the sample tube, or from label dissolved in the buffered sucrose medium were all negligible and could be contained by limiting the gain settings to a narrow range. In particular we established that at the probe concentration used in the experiments reported here, even after 24 h exposure

to the aqueous buffer, a negligible amount of dissolved label could be detected.

In these preliminary experiments we also examined the spectra of M 12-NSE in liposomes (smectic mesophases). Liposomes made from extracted membrane lipids or from dimyristoyl phosphatidylcholine, when spin labelled by the method we have described, showed a rapid incorporation of spin probe as is commonly reported [21,22]. The spectrum of labelled liposomes displayed the characteristic line shape of a spin probe oriented perpendicular to the plane of the bilayer [17,18]. Moreover, varying the probe : phospholipid ratio from 1 : 14 to 1 : 110 did not cause any major change in the line shape of the spectrum.

Spectral variations of spin-labelled microsomes

Recent work in our laboratory has shown that three characteristics of (Na^+ + K^+)-ATPase from ox brain microsomes, namely the specific activity, the rate of ouabain binding, and the effects of temperature on these enzyme characteristics are not equivalent in untreated and detergent-extracted preparations [1, 2]. Our present experiments also indicate that when untreated or detergent-extracted microsomal preparations of (Na^+ + K^+)-ATPase are spin labelled with M 12-NSE, the differences in the spectra which are obtained reflect the variations in the pretreatment of the membranes. Fig. 1 shows a representative sample of the different types of spectra obtained from a variety of enzyme preparations.

Similar to our findings with dimyristoyl phosphatidylcholine liposomes, in the presence of a suspension of ox brain microsomes, M 12-NSE will diffuse rapidly from the glass surface into the microsomal membrane. The shortest

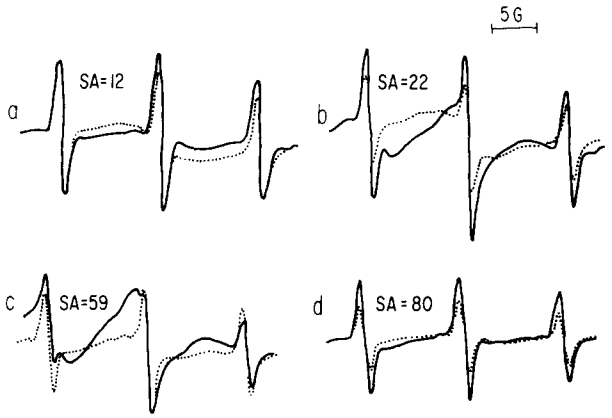

Fig. 1. M 12-NSE in ox brain microsomes. Untreated ox brain microsomes (a,b,c) and after 2 min extraction with 0.1% sodium deoxycholate in the presence of 2 mM ATP and 80 mM NaCl (d) were labelled in sample tubes containing 2 nmol M 12-NSE. Diffusion of the probe was allowed to proceed for at least 1 h prior to spectral examination. Two membrane concentrations from each of the four enzyme preparations were examined. The specific activities (S.A.) of the preparations were, respectively 12, 22, 59 and 80 μmol P_i/mg protein per h as shown on the figure. The membrane concentrations in μg protein/sample were as follows, in each set of data the lower concentration is given as the broken line: a, 1.25 (. . . . . .) and 2.5 (———); b, 4.0 (· · · · · ·) and 16.0 (———); c, 6.6 (· · · · · ·) and 21.0 (———); d, 2.6 (· · · · · ·) and 25.5 (———).

exposure time that was examined was 2 min at 37°C, after which time no further diffusion occurred. In Fig. 1a, a 2-fold difference in membrane concentration is compared. In these experiments both spectra have three sharp peaks equally spaced and of similar intensity, not unlike the isotropic spectrum of a rapidly tumbling nitroxide label in a solvent of low viscosity [6]. This type of spectrum, for which rotational correlation times can readily be determined [14—16], is characteristic of relatively unrestricted labels in the membrane. The lines in the two spectra have very similar intensities, but the low membrane concentration has some distortion of the base line due to residual M 12-NSE on the surface of the sample tube. In Fig. 1b, a 4-fold difference in concentration of membrane protein is compared. Here again the two spectra are similar in appearance and resemble the isotropic spectrum described above. There is, however, a difference in the two spectra. A broad absorption, particularly between the low-field and mid-field peaks, is apparent in the spectrum at the higher membrane concentration. This phenomenon is only observed at the lower probe : membrane ratio ($1.2 \cdot 10^{-4}$ μmol M 12-NSE : 1 μg protein) which suggests that the increase in membrane concentration (16 μg protein/sample) is responsible for the alterations in spectral line shape.

Fig. 1c shows a pair of spectra obtained with a 3-fold difference in membrane concentration. Although this concentration difference is intermediate to the two previous pairs of spectra, the divergence in spectral line shape is by far the greatest. At the lower membrane concentration (6.6 μg protein) the spectrum is typically isotropic, but when the membrane concentration in the sample is raised to 21.3 μg protein the spectrum is no longer isotropic. The form of this spectrum suggests the presence of two probe-binding sites, one of which is highly mobile (isotropic spectrum) and one where the labels are restricted (broad spectrum). Finally, in Fig. 1d we show a pair of spectra that were obtained by spin-labelling microsomes that had been extracted with sodium deoxycholate but in the presence of sodium and ATP [2]. The line shapes of both spectra are typically isotropic despite the fact that the membrane concentration of the two samples differs by an order of magnitude.

It is clear that the observed spectral variations are markedly influenced by membrane concentration. However, if the first three pairs of spectra in Fig. 1 are examined collectively, it is apparent that in addition to the membrane concentration, the specific activity of the enzyme preparations also influences the spectrum. In addition, the spectra shown in Fig. 1d indicate that treatment of the microsomes with detergents also produces changes in the spectral shape.

The effect of membrane (protein) concentration on the spectrum

To further characterize the effects of membrane concentration on spectral type we have examined the spectra of samples containing a fixed amount of probe (2 nmol) and varying amounts of membrane from a single enzyme preparation. A selection of these spectra is shown in Fig. 2. Although the spectra again vary with the membrane concentration, they can be grouped into three categories. At the low membrane concentrations the spectrum is a typical isotropic spectrum characteristic of unrestricted labels. At the very high membrane concentrations, the spectrum is considerably broadened and now resembles the line shape of the spin-labelled dimyristoyl phosphatidylcholine lipo-

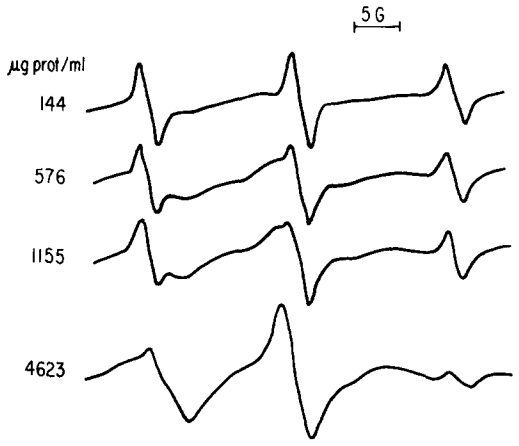

Fig. 2. The effects of membrane concentrations (μg protein) on the spectrum of microsomes labelled with M 12-NSE. Increasing concentrations of untreated ox brain microsomes were added to sample tubes each containing 2 nmol of M 12-NSE. Diffusion of the probe was allowed to proceed as described for Fig. 1. Note the change in spectrum from sharp to broad as the concentration of the membrane increases.

somes. At intermediate membrane concentrations, the shape of the spectrum is a superposition of the spectra at the extremes, with the relative intensities being determined by the membrane concentration. Thus at low membrane concentrations, the probe binds to sites that permit greater motional freedom, whereas at high membrane concentrations a site that restricts the motion of the nitroxyl is preferred. We define these two spectra as "unrestricted" and "restricted", respectively. The exact ratio of probe : membrane at which a restricted spectrum is obtained is constant for any one microsomal preparation, but may vary from one preparation to another. This latter variation bears some correlation to the degree of purification and specific activity of the enzyme and further evidence for this claim is provided later.

Spectra which are intermediate to the two limiting types described above, are a separate group constituting a mixture of two spectral types. They show the three peaks which are characteristic of the three sharp line spectrum and also display evidence of intermediate peaks. At present these mixed spectra cannot be characterized further. Presumably they arise if both restricted and unrestricted labels contribute to the spectrum.

The effect of 2 mM ascorbic acid on the two sites was also examined. For the unrestricted labels, 50% reduction in peak height intensity had occurred within the first 10 min. This is twice as long as the rate observed for the reduction of M 12-NSE when present as a solution in methanol. In contrast, after 90 min the peak height intensity for restricted spectra had decreased by less than 10%. This differential reduction by ascorbic acid has been reported by others and is believed to reflect the availability of the nitroxyl group for reduction [11—13].

The effect of enzyme purification on the spectrum

The ESR data described above strongly suggest a correlation between enzyme purity and the ESR spectrum. Furthermore, we have previously shown

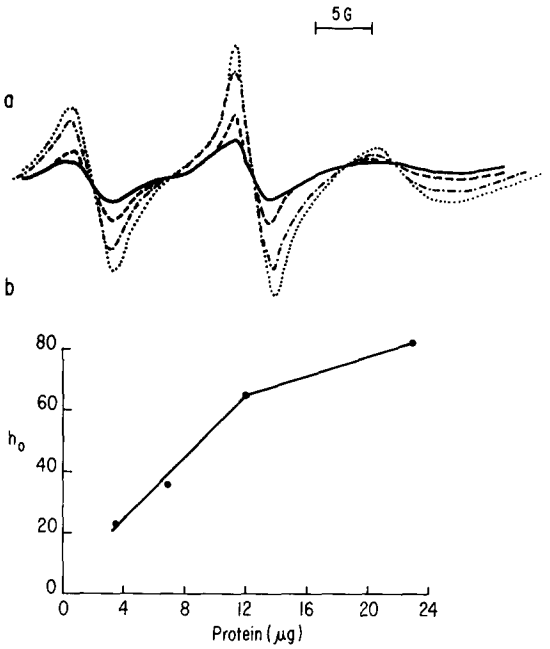

Fig. 3. The effects of membrane concentration after enzyme purification. Increasing concentrations of sodium dodecyl sulfate-extracted ox brain microsomes were added to sample tubes each containing 2 nmol M 12-NSE. Diffusion of the probe was allowed to proceed as described for Fig. 1. (a) Spectral comparison of samples containing 22.8 (· · · · ·), 11.9 (· — · — ·), 6.9 (- - - - -) and 3.4 (———) μg protein per sample tube. (b) Plot of mid-field peak height (h_0) vs. protein concentration.

that ox brain microsomes when exposed to low concentrations of sodium dodecyl sulfate in the presence of ATP will undergo dramatic increases in (Na^+ + K^+)-ATPase activity [2]. Thus we have examined the spectra of sodium dodecyl sulfate-treated enzyme preparations. Fig. 3a shows the results obtained from a more active preparation (158 μmol P_i/mg protein per h) than those found after treatment with sodium deoxycholate. Four different membrane concentrations were labelled with a constant amount of 2 nmol of M 12-NSE. Although the amount of membrane added was varied from 3.4 to 23 μg protein, it is quite clear that the spectra are all of the restricted type. This supports the notion that high activity enzyme preparations have a greater proportion of restricted sites than do untreated preparations of lower biochemical activity. If the peak height is plotted against membrane concentration, Fig. 3b, a linear plot is obtained for the first three points. This linearity is good evidence for homogeneous labelling in this system and also for the loss of unrestricted probe-binding sites in sodium dodecyl sulfate-treated microsomes. The highest membrane concentration shows a flattening of the curve, presumably because the amount of M 12-NSE probe is now the limiting factor. Three sodium dodecyl sulfate-treated preparations with elevated specific activities (158, 144 and 142 μmol P_i/mg per h) were spin labelled and examined. In all cases the restricted spectrum was observed with no evidence of mixed or unrestricted spectra.

Discussion

We have described a method which permits the spin labelling of biological membranes by diffusion of the spin probe from the surface of the glass sample tube. By using M 12-NSE, "liquid lines" are avoided because the solubility of this label in an aqueous medium is very low. This method permits the use of minimal amounts of spin probe, and also avoids contamination of the sample with organic solvents.

Liposomes of dimyristoyl phosphatidylcholine or extracted membrane lipids, demonstrate the characteristic behaviour of a typical nitroxyl spin label intercalated into a lipid bilayer. The spectrum was typical of a nitroxide restricted to motion within a cone described by the methylene chain [23]. Saturation of the liposome with spin label did not alter the line shape of the spectrum. Thus the bilayers of liposomes made with dimyristoyl phosphatidylcholine or with lipid extracts of microsomes are homogeneous with respect to binding sites for M 12-NSE.

Conversely, when ox brain microsomes are spin labelled with M 12-NSE a variety of spectra may result. At high probe : membrane ratios the spectrum is isotropic, resembling that of a nitroxide tumbling freely in solution. Presumably such a spectrum must arise from nitroxides residing at unrestricted sites. At the low probe : membrane ratios the spectrum becomes broadened and now bears similarity to that of spin-labelled liposomes. In this situation the nitroxide is in a location that partially restricts its motion. These two limiting cases are quite distinct and are best interpreted as originating from spin probes at two independent sites, an unrestricted motion site, and a restricted motion site. The spin labelling of the two sites with M 12-NSE is clearly influenced by the probe : membrane ratio, and the evidence suggests that although the restricted sites are fewer in number they possess greater affinity for the M 12-NSE.

The effect of membrane concentration on the spectrum varies from one enzyme preparation to another, and this variation can be correlated with the degree of purification of the membrane-bound $(Na^+ + K^+)$-ATPase. As the enzyme is purified the activity increases and this results in a loss of unrestricted sites with a corresponding increase of restricted sites. However, it is possible to obtain a restricted spectrum from a preparation with low specific activity, if the probe : membrane ratio is greatly reduced. Presumably an unrestricted spectrum does have a weak underlying component from restricted sites.

The spectra of sodium deoxycholate-extracted microsomes are influenced by membrane concentration, and in this respect are qualitatively similar to those of untreated microsomes. Brief exposure to sodium deoxycholate in the presence of ATP and Na^+ resulted in an elevation of the specific activity of the enzyme, apparently without a marked reduction in the number of unrestricted sites. By contrast, extraction with sodium dodecyl sulfate resulted in an increase in specific activity which was accompanied by a decrease in unrestricted sites. Presumably, under the conditions employed by us, this detergent interacts with proteins and also with lipids, as has been previously reported [24]. Preliminary examination shows a marked increase in lipid : protein ratios after extraction with sodium dodecyl sulfate compared to either untreated or sodium deoxycholate-extracted preparations.

The following summary characterizes the two sites that we have defined as unrestricted and restricted for the non-covalent binding of M 12-NSE to ox brain microsomes. (1) Two types of spectra may be obtained when ox brain microsomes are spin labelled with M 12-NSE; (2) These two spectral types are due to two different binding sites; (3) These two binding sites differ in their affinity for the spin probe; (4) The low affinity site is more abundant in crude preparations and is easily removed. The high affinity sites increase in concentration as the enzyme undergoes purification; (5) The two sites are quite distinct in their sensitivity to ascorbic acid, the low affinity site showing a considerably greater rate of reduction by this agent.

Since the reporter molecule M 12-NSE is a lipid probe, it is a reasonable assumption that the two sites reflect two different lipid domains. Although specific localization is not possible at present some comment is warranted. The domain of the unrestricted site, if it is in the core of the bilayer is a region of very great fluidity. Using the method of Kivelson [15], we have calculated tumbling times which range from $1.4 \cdot 10^{-10}$ s for a typical isotropic spectrum to $1.9 \cdot 10^{-10}$ s for an isotropic spectrum with distinct evidence of restricted labelling (i.e. presence of secondary peaks). These values are similar to the tumbling times we determined for the water-soluble stearic acid probe 5-NS in 0.25 M sucrose buffer; $1.2 \cdot 10^{-10}$ s. A lipid domain in the core of a membrane with similar viscosity to 0.25 M sucrose does not seem likely. However, typical isotropic spectra such as those described in this work, have been described for the lipid core of a smectic bilayer in the liquid crystalline state [16]. An alternative location would be the membrane-water interface. Such a position would comply with the rapid reduction of the spin label by ascorbic acid, but would require a bending of the polymethylene chain as described by Cadenhead et al. [25].

An intramembrane domain for the restricted sites is a definite possibility because of their similarity to the probe binding sites of liposomes. The correlation between restricted sites and specific activity of $(Na^+ + K^+)$-ATPase implies a lipid specificity for the enzyme protein, similar to the "liquid clustering" reported by Lee et al. [26]. Presumably the $(Na^+ + K^+)$-ATPase macromolecule, when present in ox brain microsomes is capable of selective aggregation of a phospholipid cluster of its own preference, not unlike the lipid selectivity displayed by the $(Mg^{2+} + Ca^{2+})$-ATPase macromolecule from sarcoplasmic reticulum [29].

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