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MOBILITY OF SPIN-LABELLED SULFHYDRYL SITES IN EXCITABLE TISSUE*

GREGORY J. GIOTTA and HOWARD H. WANG

Division of Natural Sciences, University of California, Santa Cruz, Calif. (U.S.A.)

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SUMMARY

The spin-label 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was shown to be attached to sulfhydryl groups of the walking leg nerve from the lobster *Homarus americanus*. Its ESR spectra indicated that it was in a highly immobilized environment. Removal of 90% of the phospholipid by chloroform–methanol extraction had no effect on the degree of immobilization. The ESR spectra of lipid extracted nerves or homogenized nerves showed marked increases in mobility of the spin label when subjected to urea, guanidine·HCl, pH, temperature, proteases, and a smaller shift in response to changes in monovalent cation concentrations. The results are interpreted as a protein conformational shift resulting in increased mobility of the spin-labelled site.

INTRODUCTION

Various functions of biological membranes have been found to be affected by blocking of membrane sulfhydryl groups. Ion transport[1] and ATPase activity[2], as well as the action potential of the squid giant axon[3] show varying degrees of inhibition as a result of treatment with mercaptide forming agents. These findings suggest a central role played by sulfhydryl containing proteins in the regulation of transmembrane ionic movements[4]. Thus, it would be of particular interest to study the properties of these sulfhydryl sites under various physiological and biochemical conditions.

The spin-labelling technique, as pioneered by McConnell and McFarland[5], and Jost *et al.*[6], has been used extensively to study biomembranes[6–10]. Inferences concerning the molecular properties of the spin-labelled sites can be made from a knowledge of the molecular motion, solvent environment, or orientation of the spin-label.

Previous workers utilizing this technique have reported binding of spin labels to sulfhydryl groups in proteins[11,12] and in proteins of non-excitable membrane preparations[13–15].

In this report, we have used the spin probe 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, an analogue of *n*-ethylmaleimide, to detect mobilities of sulfhydryl sites in the walking leg nerve of the lobster *Homarus americanus*. Changes in mobility are interpreted as the result of a protein conformational shift.

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MATERIALS AND METHODS

Biological

Lobsters (*H. americanus*) were obtained from the Great Atlantic Lobster Co. (Oakland) and kept in salt water tanks until used. The walking leg nerves were carefully dissected out, washed in ice cold Ringer solution (457 mM NaCl, 25 mM $CaCl_2$, 10 mM KCl, 4 mM Na_2SO_4, 8 mM $MgCl_2$, pH 6.8) and finally suspended in 5 ml Ringer solution. If the axons were to be homogenized after spin labelling they were put in 10 ml of Ringer solution and homogenized in a motor-driven glass–teflon homogenizer for 5 min at 4 °C. Next the whole homogenate was spun down at 20000 $\times g$ for 20 min.

Chemicals

(a) 3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (Syva Associates) was added to the Ringer solution containing the lobster nerves to make $5 \cdot 10^{-4}$ M, and the preparation was agitated while allowed to come to room temperature over 3 h. Next, the nerves were washed exhaustively with cold Ringer solution.

(b) The sulfhydryl blocking agents *n*-ethylmaleimide and *p*-chloromercuribenzoate were obtained from Calbiochem. $HgCl_2$ was of analytical reagent grade (Mallinckrodt). The amino group specific reagent 2-methoxy-5-nitrotropone was from Calbiochem. Prior to spin labelling, the blocking agents were incubated with the lobster axons at a concentration of $5 \cdot 10^{-4}$ M in Ringer solution for 12 h at 4 °C followed by a wash with Ringer solution.

(c) The following salts were of analytical reagent grade (Mallinckrodt): NaCl, KCl, LiCl, $MgCl_2$, $BaCl_2$ and $CaCl_2$. After lipid extraction, spin-labelled nerves were placed for 15 min in the appropriate salt solution in 1 mM Tris–HCl buffer at pH 7.2. The salt solutions were checked before and after use to insure that the pH remained constant.

(d) Sodium dodecyl sulfate was obtained from Sigma and recrystallized from 80% ethanol. Urea and guanidine·HCl were supplied by Mallinckrodt and Sigma (grade 1), respectively. Sodium dodecyl sulfate, urea and guanidine·HCl were made up in 0.05 M phosphate buffer (pH 5.6). Dioxane was of spectrograde quality.

(e) Creatine kinase, bovine serum albumin, and pronase were supplied by Calbiochem. Trypsin was from Worthington. Creatine kinase and bovine serum albumin were spin-labelled according to Griffith and McConnell[11].

(f) The following buffers were used: pH 1, HCl–KCl; pH 3,5 and 7, citrate; pH 9, boric acid; pH 10.6, glycine–NaOH; and pH 5.6, phosphate. All were used at a concentration of 1 mM, except for the phosphate which was 0.05 M.

(g) Temperature studies were carried out by sealing a segment of nerve in a capillary tube containing phosphate buffer (0.05 M, pH 5.6). The tube was placed in the Dewar Varian temperature accessory and the spectra recorded at 10 °C intervals.

In all studies involving whole nerves, the ESR spectra were recorded by tying the nerve at one end with a piece of string and gently pulling it through a cylindrical glass tube constricted at the center. In studies using nerve homogenates, the samples were read in capillary tubes sealed at both ends.

All spectra were recorded on a Varian model E-3 EPR spectrometer.

Lipid extraction and assays

Complete lipid extraction was done according to the procedure of Folch[16]. Total phosphorus was determined by the method of Bartlett[17], and taken to be a realistic estimate of the amount of phospholipid. ATPase was assayed by the method of Camejo *et al.*[18]. Briefly; lobster nerves were removed and placed in 0.25 M sucrose buffered with Tris–HCl, pH 7.4. They were homogenized in a motor-driven glass–teflon homogenizer at 4 °C. Next, 0.5-ml aliquots were distributed to all but one tube which had 0.5 ml sucrose buffered with Tris. Another tube had just homogenate, and two others had added to them enough *N*-ethylmaleimide or spin label to make 1 mM. All assays were done in triplicate. Next, 1.5 ml of a reaction mixture which contained 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP (Tris salt, Sigma), and 30 mM Tris–HCl (pH 7.4) was added to each tube and kept at 37 °C for 35 min. The reaction mixture prior to addition was preincubated in a water bath at 37 °C for 10 min. At the end of 35 min, 1 ml of 6% $HClO_4$ was added to each tube. The precipitated protein was spun down and the supernatant saved for the phosphate determination.

Spectral measurement

The existence of two populations of sulfhydryl bound spin labels in lobster membrane is indicated by the presence of both peaks 1 and 2 in Fig. 1c. In order to measure changes in mobility the ratio of Peak 2 to Peak 1 was used (Fig. 1). An increase in this ratio is best interpreted to mean that all spin-label sites do not have the same mobility and that the more mobile population increases at the expense of the immobile sites. Peak 1 appears to arise from spin labels that are totally or almost totally immobilized, and that Peak 2 originates from a population of sulfhydryl bound spin labels that are in an environment permitting rapid tumbling. For a given experimental condition, the two populations of spin labels are probably separated in space such that they may be in completely different local molecular environments.

Another spectral parameter which is related to the mobility of the spin label is the separation of the outer hyperfine extrema. In all cases, except for the cation effects, an increase in the Peak 2 to Peak 1 ratio was also accompanied by a detectable decrease in the separation of the outer hyperfine extrema. Thus, the ratio of Peak 2 to Peak 1 represents a rough estimate of the overall mobility of all the spin-labelled sites. This method of interpretation was used previously by various authors[12,14].

The results of all experiments were repeated at least 5 to 7 times and were reproducible.

RESULTS AND DISCUSSION

The ESR spectra of the spin-labelled nerve after exhaustive washing with Ringer solution displayed two components: a very intense solution spectrum that was superimposed on a strongly immobilized spectrum. The former component was easily observable at low gains while the latter was observable only at high gains (Fig. 1, Curves A and B). In most of our experiments, spin-labelled nerves were subjected to lipid extraction by soaking in two changes of chloroform–methanol (2:1, v/v) for 30 min each and then washed with 5 mM Tris–HCl buffer (pH 7.2). This procedure was found to remove 90% of the phosphorus present in the nerve bundles. The ESR spectrum of such defatted nerves showed that the chloroform–methanol

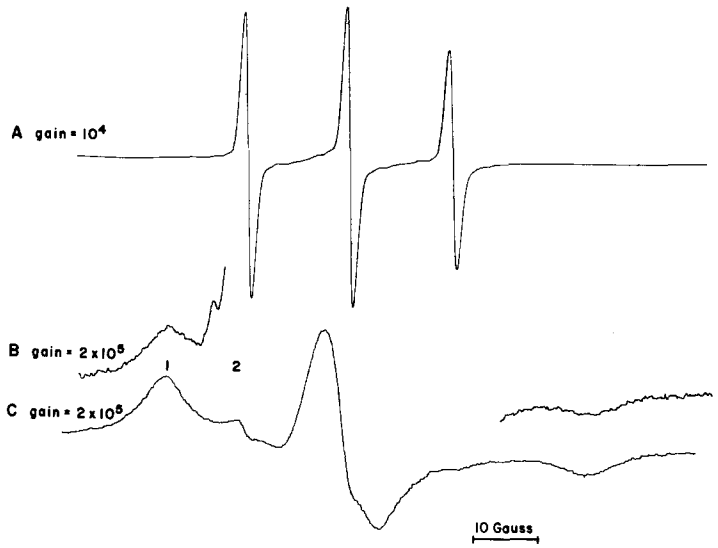


Fig. 1. ESR spectra of lobster walking leg nerve labelled with 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl. A and B, untreated; C, treated with chloroform–methanol.

treatment removed the mobile component, leaving only the strongly immobilized portion of the spectrum (Fig. 1, Curve C). Instead of the chloroform–methanol treatment, the mobile component of the ESR spectrum could also be revoved by homogenization of the spin-labelled nerve followed by washing; the results were similar.

The mobile component

Since it is not the purpose of this paper to deal with the mobile component, it is sufficient to include some brief observations. The intense solution spectrum removed from chloroform–methanol treated nerve or homogenized nerve was detected in the chloroform–methanol and wash solutions. This spectrum appears to arise primarily from spin labels bound to a small molecular weight substance, as determined by gel filtration on a Sephadex G-15 column and by the fact that the substance was dialyzable.

Although the free-spin label is initially permeable to the plasma membrane, once it is bound to this small molecular weight substance (*e.g.* glutathione, *etc.*) in the cytoplasm, the label is no longer able to diffuse out of the nerve membrane even with exhaustive washing. Chloroform–methanol treatment or homogenization breaks down the membrane barrier, thus releasing this spin-labelled substance. Our findings are consistent with those of Sandberg *et al.*[13] on whole and ghost erythrocyte preparations.

The strongly immobilized spectrum

The presence of Peak 2 in Fig. 1, Curve C shows the spectrum is a composite of strong and weak immobilization. Thus the probe is labelling sites that give it different degrees of freedom.

The splittings between the two extreme peaks of the immobilized spectra show no difference between fresh and chloroform–methanol treated nerves (Fig. 1, Curves B

and C). Such results indicate the lack of drastic structural alteration of these spin-labelled sites in defatted nerves.

The spin-labelled site was identified using highly specific sulfhydryl and amino group reagents. Pretreating fresh lobster nerves with sulfhydryl blocking agents, such as *p*-chloromercuribenzoate, $HgCl_2$, or *n*-ethylmaleimide, all at $5 \cdot 10^{-4}$ M for 12 h prior to the addition to the spin label resulted in the complete absence of the spectrum in Fig. 1, Curve C. These results are similar to those found for erythrocyte ghost membrane[13,14]. Pretreating of the lobster nerve with 2-methoxy-5-nitrotropone, an amino group-specific reagent at pH 8.2 (ref. 19), had no effect on the spectrum. The ratio of Peak 2 to Peak 1 (Fig 1, Curve C) remained constant even with exhaustive washing. These results indicate that the entire ESR spectrum as shown in Fig. 1, Curve C resulted from covalent binding of the spin label to sulfhydryl groups.

Since it is known that membrane bound ATPase activity is reduced by *n*-ethylmaleimide, it was desirable to see if the spin-labelled analogue acted similarly. In agreement with Skou and Hilberg[2], membrane bound ATPase activity of non-chloroform–methanol treated homogenate was shown in our system to be reduced 50% by *n*-ethylmaleimide. Similarly, the spin-label analogue also reduced ATPase activity by 50%. Thus the spin label is probably acting in a functionally analogous manner upon binding to the axonal membrane.

To further characterize the binding site, lipid-extracted axons were incubated at 37 °C in trypsin at a concentration of 1 mg/ml in lobster Ringer solution. At the end of 40 min, trypsin had reduced the strongly immobilized spectrum of the spin-labelled nerve to 50% of its original intensity and free solution ESR signals were subsequently detected in the incubating solution. Dialysis of this incubation solution against Ringer solution at 4 °C was incapable of removing the ESR signal which was completely removed following exposure to pronase (1 mg/ml).

Tobias[20] has shown that trypsin, after prolonged incubation (4 h), can enter the lobster giant axon without appreciable functional inactivation. Since trypsin is known to destroy the action potential when applied internally to the axon[21], we may infer that the quantity of trypsin entering the axon is minimal at the end of 4 h. If this inference is correct, we may assume no appreciable trypsin entered the lobster axons at the end of 40 min in our experiments. Our results would then suggest that approx. 50% of the spin-labelled sites were attacked by trypsin on the external membrane surface.

Effect of protein denaturation

It was hypothesized that the spin-labelled sulfhydryl sites are located in protein crypts which restrict the mobility of bound spin labels. To test this idea, a variety of denaturants were tried.

Temperature

As shown in Fig. 2, the defatted nerves showed gradual increases in the ratio of Peak 2 to Peak 1 over a range of 25 to 65 °C, above this there are greater increases in the ratio of Peak 2 to Peak 1. Below 70–75 °C the temperature effects are reversible, above this temperature the nerves maintained an irreversible mobile spectrum. Creatine kinase and bovine serum albumin, both of which contain restricted sulfhydryl sites[11], showed similar results as the defatted nerves under analogous conditions.

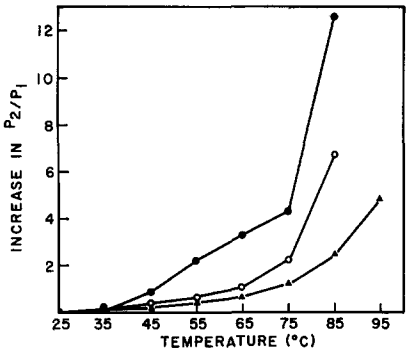


Fig. 2. Changes in mobility of spin-labelled axon (▲—▲), creatine kinase (○—○), and bovine serum albumin (●—●) as a function of temperature.

Urea and guanidine·HCl

Soaking spin-labelled nerves (either fresh or chloroform–methanol treated) in 10 M urea or 6 M guanidine·HCl for 15 min resulted in the complete conversion of the immobilized component into the mobile component (Fig. 3). Again, using the

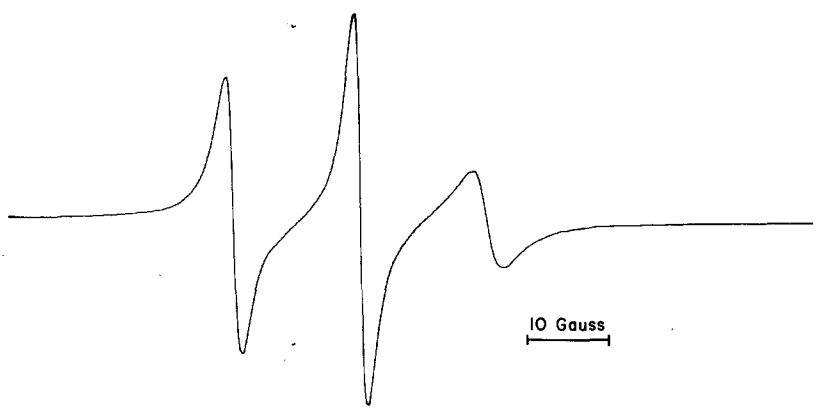
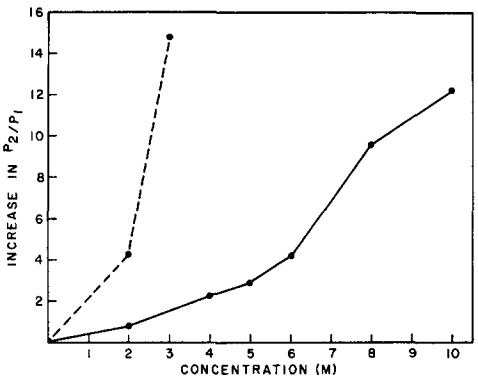


Fig. 3. ESR spectrum of a spin-labelled nerve after soaking in 10 M urea. 6 M guanidine-HCl gave a similar spectrum.

ratio of Peak 2 to Peak 1 as an indication of mobility, the effects of different concentrations of urea and guanidine·HCl are shown in Fig. 4. The latter agent was capable of exerting greater increases in mobility at considerably lower concentrations than urea. It is unlikely that either compound is acting as a solubilizer since the effects of both agents can be completely reversed by washing the nerves in phosphate buffer for 5 to 10 min.

Dioxane

Since both urea and guanidine·HCl disrupt secondary as well as tertiary structures of protein, we investigated effects of another class of denaturants. Proteins denatured with dioxane have been shown to have a certain degree of ordered conformation *i.e.* a high proportion of α-helix[22]. Grigorian *et al.*[23] used dioxane to ob-



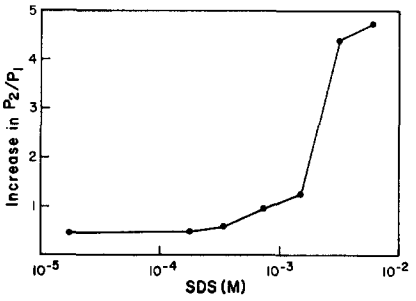


Fig. 4. Plot of mobility *versus* concentration of urea (——) and guanidine·HCl (– – –).

Fig. 5. Effect of sodium dodecyl sulfate (SDS) on the mobility of the sulfhydryl-bound spin label.

serve changes in the mobility of spin-labelled bovine serum albumin and interpreted these changes to be due to the destruction of tertiary foldings of the protein. In our experiments, dioxane up to a concentration of 60% had no effect on the mobility of the sulfhydryl-bound spin labels. This finding is consistent with our initial observation that the spin-labelled site showed no response when subjected to chloroform–methanol treatment. Our results suggest two possibilities concerning the mobility of the spin probe: (1) the probe is not immobilized by tertiary structures, or (2) unlike bovine serum albumin, the tertiary structure of our membrane protein is minimally affected by dioxane.

Effect of detergent

Sodium dodecyl sulfate was tried over a range of $1.8 \cdot 10^{-5}$ to $6 \cdot 10^{-3}$ M. As shown in Fig. 5 there was little effect until the critical micelle concentration of the detergent[24] was reached at approx. $3.5 \cdot 10^{-3}$ M, above this sodium dodecyl sulfate exerted little additional effect. Exhaustive washing was incapable of reversing the detergent effects. Since 90% of the lipid fraction of the axon was not present, the increase in mobility was presumably due to the interaction of the detergent with the spin-labelled protein.

Effect of pH

The effects of pH on the spin-labelled nerve showed a minimum in mobility over the range of 3.5 to 5.5 (Fig. 6). On either side of this minimum the mobility showed dramatic increases. This observation is in agreement with the results of Chapman *et al.*[15] on the erythrocyte membrane. Since the pH range of 3.5 to 5.5 gives a minimum in mobility, and since this range corresponds to the isoelectric point of most membrane proteins[15], the decrease in mobility may be due to the lack of charge repulsion which would enable the spin-labelled protein to form a tighter complex about the spin probe.

Effect of cations

Monovalent cations stimulated a progressive increase in mobility of the sulfhy-

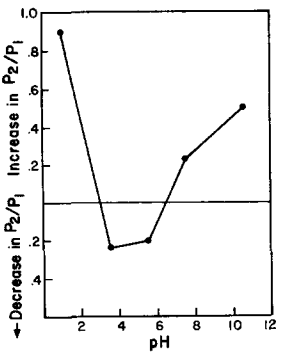


Fig. 6. Plot of mobility *versus* pH.

dryl-bound spin labels over a salt concentration of 10^{-3} to 10^{-1} M. The maximal response was observed at close to physiological concentrations of 10^{-1} M. The effects of LiCl (Fig. 7) are chosen to illustrate this observation. The order of increasing mobilizing effect was $K^+ > Li^+ > Na^+$. Washing the nerve in salt free Tris buffer reversed the effect.

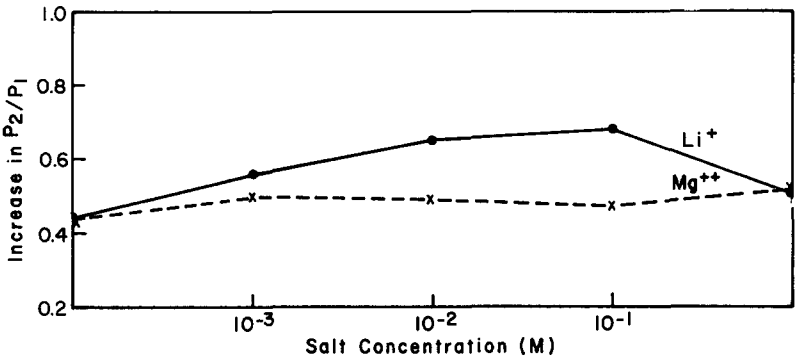


Fig. 7. Effect of LiCl and $MgCl_2$ on the mobility of 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl bound to lobster leg nerves.

Over the same concentration, divalent cations (Mg^{2+}, Ca^{2+}, Ba^{2+}) showed no effect. The divalent cations are represented by Mg^{2+} in Fig. 7. Bathing the spin-labelled nerve in either monovalent or divalent cations above 1 M resulted in a decreased mobility of the sulfhydryl bound spin label.

Whether the monovalent cation mobility increase is due to a general salt effect, or represents a specific salt-protein interaction involved in a physiological reaction is not clear. However, it is obvious that H^+ and divalent cations do not affect the sulfhydryl mobility as do Na^+, K^+, and Li^+.

CONCLUSION

As discussed in the results section, the blocking experiments showed the probe to be attached to sulfhydryl-containing proteins. The spin probe was shown to reduce

membrane ATPase activity by 50%, and simultaneously gave a strongly immobilized signal in nerve homogenates. These observations suggest the spin-labelled protein to be membrane bound. Since the activity of ATPase is reliant on free sulfhydryl groups, at least part of the ESR spectrum is due to the probe binding to the enzyme. It is probable, however, since the mobility of the sulfhydryl sites is not uniform, that the probe was also bound to proteins other than ATPase. These proteins are sensitive to denaturants. Furthermore, trypsin was capable of releasing spin-labelled peptide fragments and simultaneously causing a 50% reduction in the strongly immobilized peak. Of particular interest, in light of theories postulating an interaction of Na^+ or K^+ with an electrogenic protein[4], was the increase in mobility exerted by monovalent cations.

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