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PHOTOAFFINITY SPIN-LABELING OF THE Ca^{2+}ATPase IN SARCOPLASMIC RETICULUM : EVIDENCE FOR OLIGOMERIC STRUCTURE

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SUMMARY : A spin labeled fatty acid (16-doxylstearic acid) was linked to a photochemical reacting group (azido derivative). When the molecule is introduced, at a low concentration, into rabbit sarcoplasmic reticulum membranes, the spectrum before illumination is identical to the spectrum obtained with the corresponding spin labeled fatty acid. After illumination, a large immobilized component is seen.It corresponds to about 70% of the ESR signal of the effectively bound label, at room temperature. The fraction of immobilized component varies with temperature,from 100% at 0°C to 50% at 35°C.Addition of a small amount of detergent (dodecyl octaethylene glycol monoether), under non solubilizing conditions,decreases the fraction of signal due to a strongly immobilized probe. A possible interpretation is that the immobilized signal reflects protein bound spin labels trapped in Ca^{2+}ATPase oligomers, which are partially dissociated by detergent addition or temperature increase.

INTRODUCTION :

The physical state of the lipid chains in contact with intrisic proteins can be studied by ESR. Different laboratories following the work of Griffith's group have used spin labeled fatty acids or phospholipids in membranes or reconstituted systems (1). This technique has the disadvantage that only a small fraction of the probes are at the lipid-protein interface under normal physiological conditions.Concurrently, we have developped a different approach to study lipid-protein interactions with spin-labels. In order to increase the affinity of the labeled hydrophobic chains towards membrane proteins we have synthesized either fatty acids linked to specific protein ligands (2) or fatty acids coupled to a sulfhydryl reagent such as a maleimide (3) or an isocyanate residue (4). This latter technique however relies on the presence of receptor sites or SH groups suitably positioned on the proteins. In order to be less dependent on these specific sites, we have now synthesized molecules having a photochemical reacting group on the polar moiety of an amphiphilic

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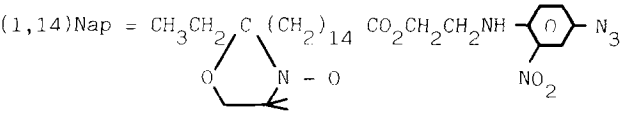
spin label. Thus it is possible, in principle, to attach a labeled chain at the hydrophobic interface of any intrinsic protein. The present report shows preliminary results obtained with a spin label of this type bound to Ca^{2+} ATPase in purified sarcoplasmic reticulum.

MATERIALS AND METHODS :

Spin label synthesis : 2-(2-nitro-4-azido-phenyl) amino ethyl 16-doxylstearate [(1,14) Nap] was synthesized by esterifying the mixed anhydride of the spin-labeled 16-doxylstearic acid and ethylchloroformate with 2-(2-nitro-4-azido-phenyl) amino ethanol (A). The latter was obtained by reaction of 2-amino ethanol with 1-fluoro-2-nitro-4-azido benzene, itself prepared by the procedure of FLEET et al (5).



Synthesis of (A) ; A mixture of 1 mmol of fluoronitroazidobenzene, 1 mmol dry amino ethanol, 2 ml of dry dimethylsulfoxide and 420 µl of dry triethylamine, according to LEVY (6), was heated at 60°C, during 16 h, in a sealed glass protected from day light. All following procedures were performed under red light. The solution was then poured into 20 ml water containing 13 mmol sodium hydroxyde. After extraction with diethyl ether (5 x 5 ml) and drying the organic layer with magnesium sulfate, the solvent was removed, the product deposited on 1 g silica gel and chromatographed on 10 g dry packed silica gel. Elution with ether gave a first moving band which was collected, affording 138 mg (62%) of (A) : single spot on analytical thin layer chromatography (silica gel, Et_2O) with Rf = 0.48 ; visible absorption (ethanol) at 456 nm (ε = 5800).

Synthesis of 1,14 Nap : 64 µmol of spin labeled fatty acid prepared by the procedure of HUBBELL and Mc CONNELL (7) was converted into the mixed carboxylic carbonic anhydride according to FAVRE et al. (3). To a solution of this latter in 3 ml of dry toluene were added 30 µl of dry triethylamine and 62 µ mol of compound (A). The mixture was heated at 50°C, for one day, in a sealed glass protected from day light. After removal of toluene, the residue was puridied by preparative thin layer chromatography on a 20 x 20 x 0,2 cm silica gel G 60 (Merck) plate eluted with diethylether:hexane:triethylamine (7:3:1). The band at Rf = 0.54 was scrapped off and extracted with diethylether, affording 16.5 µmol (26%) of the photoaffinity spin labeled ester (1,14) Nap : single spot on analytical thin layer chromatography (silica gel, Et_2O) with Rf = 0.85. Visible absorption shows a maximum at 448.5 nm (ε = 5350).

Membrane preparation and control : Sarcoplasmic reticulum from rabbit skeletal muscle was prepared as previously described (8) and freed of proteins other than (Ca^{2+} + Mg^{2+})-ATPase by the method 2 of MEISSNER et al. (9). The membranes were stored in the presence of 0.3 M sucrose, 1 mM HEPES pH 7.5 at -20°C. This preparation gave a single band on gel electrophoresis (10). Ca^{2+} dependent ATP hydrolysis in the various ATPase preparation was measured spectrophotometrically at 20°C by a NADH coupled assay (11). The enzyme activity was usually 3-4 µmol ATP/mg protein/min. The detergent used in some experiments was dodecyloctaethylene-glycol monoether ($C_{12}E_8$), purchased from Nikko Chemical, Tokyo.

Spin labeling : (1,14) Nap was dissolved in ethanol and added under dim red light to 200 µ l of membrane sample containing 16 mg protein/ml, the percentage

of ethanol in this medium being kept below 1 %. After a 30 minute incubation time at 37°C, the preparation was sonicated in the dark for 15 seconds in order to avoid label micelles (Ultra Son, Annemasse, small tip, low power). The ration of spin labels to proteins in the final medium was 0.35. The sample was then concentrated to 40 mg protein/ml by centrifugation in a buffer : 10^{-1} M KCl, 10^{-2} M HEPES pH 7.5, 10^{-4} M $CaCl_2$. (HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid).

The label containing membranes were illuminated 30 seconds with a laser beam (Spectra Physics, model 164, ionized argon, 1 W, 488 nm).

Washing of the sample with bovine serum albumin in order to remove non-bounded label from the membranes after illumination was performed on some samples.

In one case, after ESR measurements, lipids were extracted by the method of SCHMID et al (12) and proteins were recovered as a powder after lyophilisation. Quantitative determination of the amount of spin label in the two fractions was then carried out.

Addition of detergent ($C_{12}E_8$) : $C_{12}E_8$ can solubilize sarcoplasmic reticulum membranes when added at a concentration greater than 2 mg of detergent/mg protein (13). Aliquots of a 100 mg/ml solution of $C_{12}E_8$ were mixed with the sample in order to introduce 0.1 or 0.2 mg/mg protein into the membrane.

ESR measurements : ESR spectra were recorded on a VARIAN E109, connected to a Tektronix computer (4051) and provided with a field frequency lock (Varian) and a temperature-control accessory.

RESULTS

(1,14) Nap was introduced into liposomes made with egg lecithin (70%) and dipalmitoyl phosphoryl ethanolamine (30% mole/mole). The ESR spectrum at room temperature (20°C) is identical to the spectrum obtained with the corresponding fatty acid. If the sample is illuminated, no change in line-shape can be observed.

The results are quite different with the sarcoplasmic reticulum membranes. Here again, before illumination the spectrum is identical to the spectrum of the fatty acid spin label (dotted lines in figure 1). The full line spectrum of figure 1 shows the modification induced by illumination. The large immobilized component visible after illumination must be associated with binding of the spin label to ATPase. Indeed, if quantitative estimates are made of the fraction of spin labels associated with lipids and proteins respectively, it appears that more than 50% of the spin labels are found associated with the proteins. An accurate determination is impossible because of the difficulties arising when resuspending denaturated proteins.

The spectrum of the bound labels can be obtained by subtraction of a fraction of the spectrum before illumination from the one after illumination at the same temperature. Because the splittings of these two spectra are not identical, it is possible to determine the fraction of unbound label with a reasonable accuracy ($\pm$ 5% at worse). This fraction is found to be the same at each temperature (about 50%).

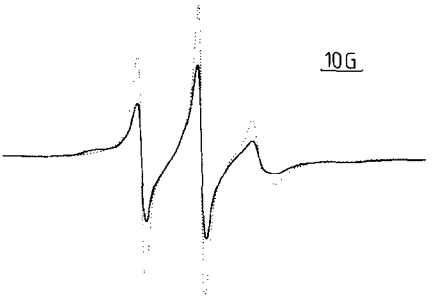


Figure 1 : ESR spectrum at 15°C of (1,14) Nap in sarcoplasmic reticulum membrane after laser illumination. The dotted curve corresponds to the spectrum recorded before illumination, with the same integral; it is identical to the spectrum obtained with a fatty acid.

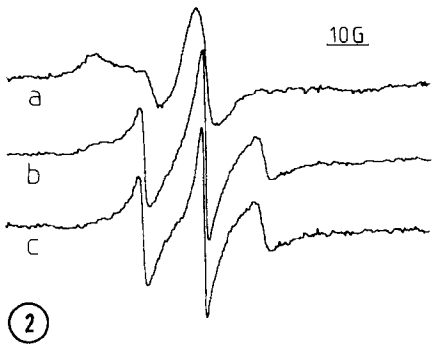
Except at 0°C, the resulting spectrum of bound label shows two components corresponding to a strongly immobilized probe and to a relatively weakly immobilized one, their ratio varying with temperature. At 0°C, we observed an almost pure "immobilized spectrum" (figure 2a). Subtraction of this latter from each spectrum of bound label gives the approximate shape and fraction of the "mobile" component at each temperature. Figures 2b and 2c show the bound spectrum and the corresponding mobile component at 35°C. This mobile component differs significantly in line width and splitting from the spectrum of unbound label in the same conditions. The proportions of immobile component were 95±5% at 5°C, 75±5% at 15°C, 60±10% at 25°C and 50±10% at 35°C respectively. The use of an immobilized component with a lower splitting at 35°C, increases slightly the fraction of the immobilized component at this temperature. However, the ratio of the 2 components appears still temperature dependent

By addition of detergent ($C_{12}E_8$) at a non-solubilizing level, an appreciable change of the ESR spectrum took place (figure 3), suggesting a decrease in the immobilized spectrum. As before, subtraction of a fraction of the spectrum of a non illuminated sample with detergent gives the spectrum of unbound label, whose analysis indicates, at 15°C, 50±10% of immobile component with either 0.1 or 0.2 g $C_{12}E_8$/g protein.

Compared to 75±5% without $C_{12}E_8$, these values indicate a rather weak but significant decrease of immobilization by the detergent. The method based on a linear combination of the spectra of bound label (14) was not useful for us probably because some slight splitting variations between mobile spectra.

DISCUSSION

The present study shows that a nitroxide can be coupled to a photoaffinity label with only a slight reduction during illumination. Hence this provides means to covalently bind a paramagnetic group to proteins. In the



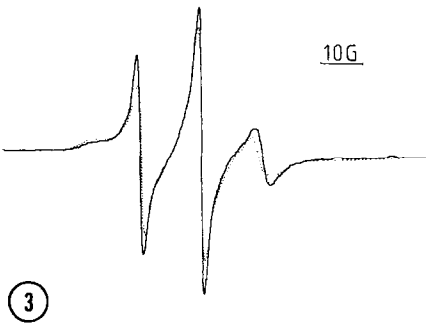


Figure 2 : ESR spectra of (1,14) Nap bound to Ca^{2+} ATPase in sarcoplasmic reticulum membrane obtained by subtracting 50% of the spectrum recorded before illumination from the spectrum obtained after illumination.
a) 0°C ;
b) 35°C ;
c) mobile component at 35°C (see text).

Figure 3 : ESR spectrum at 15°C, of (1,14) Nap in sarcoplasmic reticulum membrane, after laser illumination and incorporation of 0.2 g $C_{12}E_8$/g Ca^{2+} ATPase. The sample was washed with bovine serum albumin, which removed only a part of unbound label. Dotted curve represents the spectrum of the same sample before detergent addition.

present study the affinity is provided solely by the hydrophobic character of the fatty acid chain. The spin label can react with any protein present and to some extent perhaps also on lipids. However it is very clear from our results that a large fraction of (1,14) Nap binds to the proteins in the sarcoplasmic reticulum membranes. At least, one can detect two very different environments of the labels associated with the protein, actually with Ca^{2+} ATPase which is largely predominant in the membrane preparation (9).

Does this mean that the average hydrophobic environment of Ca^{2+} ATPase is indeed immobilized such that more than 50% of the lipid chains in direct contact with the protein are rigid ? This could fit into previously described model for lipid-protein interactions in Ca^{2+} ATPase reconstituted systems (15): the boundary lipids would pass a long time in close contact to the protein and would have their lipid chains completely immobilized. According to this model, the spectra before and after illumination should be the same. The strong immobilized component appearing after the laser illumination demonstrates that the excited molecules diffuse until they reach a protein. This is possible if the lifetime of the radical, which is less than 10^{-4} s (16), is longer than the lifetime of boundary lipids. This agrees with RICE et al (17) who showed that the average phospholipid exchange rate at the boundary of Ca^{2+} ATPase is fast. Moreover, as the (1,14) Nap prefers to bind to Ca^{2+} ATPase, the nitrene reactivity must be high towards certain protein residues such as SH groups (18).

Another way to explain appearance of an immobilized spectrum after illumination could be the existence of a few hydrophobic sites where a fatty acid chain would be temporarily immobilized. Ca^{2+} ATPase may have a hydrophobic crevice, or more likely, there may be clefts between oligomeric forms of the protein. But we have shown that an increase in temperature or a small amount of detergent can decrease the percentage of immobilized component. If these latter phenomena were simply a competition for hydrophobic chain intercalation into a protein crevice, it would be rather difficult to understand because the spin labels are covalently bound to the proteins. In contrast, the temperature and detergent effects may be to disrupt protein oligomers and hence release immobilized spin labels entrapped between proteins.

In conclusion, we do not think that the strongly immobilized signal is proof of an immobilized boundary layer. Rather it suggest that Ca^{2+} ATPase in sarcoplasmic reticulum exists under physiological conditions partially in the form of an oligomer. Indeed, the oligomericity of the Ca^{2+} ATPase in the membrane has been suggested, following the experimental results from a variety of techniques such as analytical centrifugation on the detergent solubilized protein (11,19), electron microscopy (20), fluorescence transfer (21), fluorescence anisotropy measurements (22) and laser-flash induced photodichroïsm (23). However, variation of the polymerized fraction with temperature is yet a point of controversy (23,24).

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