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A STUDY OF THE STRUCTURAL INTEGRITY OF SPIN-LABELLED PROTEINS IN SOME FRACTIONS OF HUMAN ERYTHROCYTE GHOSTS*,**

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SUMMARY

The spin label method has been applied to the problem of isolating membrane proteins without altering their structure. Spin-labelled erythrocyte ghosts were dissolved using eight different procedures, and the electron spin resonance spectra of the resulting fractions studied prior to and after removal of solubilizer. Marked differences were observed in the spectral perturbations caused by the various dissolution procedures. However, in a number of instances fractions were obtained after removal of solubilizer whose spectra resembled that of the labelled ghosts, suggesting that the structural integrity of the labelling site had been largely regained. These results are taken to indicate the fractions which are appropriate departure points for subsequent more detailed isolation studies where retention of structural integrity is of interest. The data also suggested mechanisms by which the various solubilizers operate, and that the structures of some proteins are not highly dependent on the lipids which can be extracted by *n*-butanol.

INTRODUCTION

An important question which arises when membrane proteins are isolated is whether or not structural integrity has been maintained. To answer this question one requires techniques for comparing the structures of proteins while part of a membrane with those after isolation. Although antigenic and enzymic activity are useful in this regard[1,2], other techniques are necessary because there may be membrane proteins which do not possess such readily-apparent distinctive properties[3]. A technique with the potential for making the appropriate comparison is the spin label method, which has been shown to be sensitive to protein conformational changes[4–10]. The present study uses this method to detect structural changes following dissolution of spin-labelled erythrocyte ghosts by eight different methods. One of the main objectives was to identify those methods causing the smallest or no spectral changes. The absence of ESR spectral changes after solubilization should at least indicate that the labelling site has retained structural integrity. The spin label used was *N*-(1-oxyl-

Abbreviation: Mal-6, *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)maleimide.

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2,2,6,6-tetramethylpiperidinyl)-maleimide (Mal-6), which reacts selectively with sulfhydryl groups[11,12]. The study, therefore, focuses attention on structural changes in those proteins which contain spin-labelled sulfhydryl groups.

METHODS AND PROCEDURES

Hemoglobin-free erythrocyte ghosts were prepared by the procedure of DODGE *et al.*[13]. Labelling was carried out in phosphate buffer (pH 6.8) at 21–23° by stirring gently for 1 h approx. 1 mg of Mal-6 per 10 mg of membrane protein. The labelled cells were then isolated by centrifugation at 4° and washed 3 times with 350 ml of isotonic phosphate buffer (pH 7.6). The pH 6.8 buffer was prepared by adding to a solution containing 12 mM NaH_2PO_4, 12.6 mM Na_2HPO_4, 50 mM NaCl, 50 mM KCl, 2.5 mM $MgCl_2$ and 12.5 mM disodium adenosine triphosphate, enough NaOH to bring the pH to 6.8. This composition was employed in order to protect sites involved in the Na^+-K^+-Mg^{2+}-activated ATPase activity, since these cells were also used in experiments dealing with conformational changes resulting from the action of this enzyme. The buffer was successful in maintaining more than 80% of the desired activity.

The eight solubilizing methods employed depended on the use of 0.01 M HCl (pH 2), 0.01 M NaOH (pH 12) (D. STEERS AND W. G. MARTIN, personal communication); 8 M urea[14]; 1% lysolecithin[15]; sodium deoxycholate[16]; 0.2% sodium lauryl sulfate[17]; *n*-butanol[18]; and pyridine[19]. The general procedure was to incubate the cells (1–3 mg protein per ml) in a solution of the solubilizer, record the ESR spectrum of soluble and insoluble fractions, dialyze to remove solubilizer (18–72 h at 4°), and record the spectra of the products. The soluble and insoluble fractions were isolated by centrifugation at 17000 × *g* at 4° (31900 × *g* for deoxycholate treated samples), and the insoluble fractions were washed twice in the centrifuge with appropriate solutions. Prior to adding solubilizer the cells were transferred to a hypotonic phosphate buffer (pH 7.6) and 20 mosM, to maximize solubilization, since preliminary experiments suggested this might occur in some instances at lower ionic strengths. An exception was in experiments using butanol and pyridine, where the cells were washed with cold, deionized water because of the requirement that salt be absent[18,19].

Solubilizer was added to approx. 1 ml of cell suspension as the solid (urea, lysolecithin), the pure liquid (pyridine), in the form of a concentrated solution in hypotonic buffer (sodium deoxycholate, sodium lauryl sulfate), or by dialysis (0.01 M HCl, 0.01 M NaOH). Solubilization using butanol was carried out as described by REGA *et al.*[18]. The addition of solubilizer was made at 21–23° except for experiments using pyridine, butanol, 0.01 M HCl and 0.01 M NaOH where the temperature was 4°. The extent of solubilization was measured by the amount of protein dissolved. Protein concentrations were estimated by the Folin–Lowry method[20] using crystalline bovine serum albumin as a standard. To estimate lipid phosphorus and cholesterol an aliquot of the ghost suspension or fraction thereof was dried under nitrogen and then extracted three times with chloroform–methanol (2:1, by vol.). Phosphorus was determined by the method of KING[21] and cholesterol by the method of BEST *et al.*[22]. Phospholipid content was taken to be equal to lipid phosphorus × 25. The sodium deoxycholate, sodium lauryl sulfate, *N*-ethylmaleimide, and *p*-chloromercuribenzoate employed were supplied by Mann Research Laboratories, New York, N.Y. The egg lysolecithin

was supplied by Pierce Chemical Company, Rockford, Ill. The spin label was a product of Varian Associates, Palo Alto, Calif. All other chemicals were reagent grade. ESR spectra were run at 23° on a Varian E3 spectrometer.

RESULTS

The ESR spectrum of the labelled ghosts, Fig. 1A, is similar to that obtained by other workers[23,24]. It is a composite of at least two spectra: one due to spin labels which have no independent motion relative to the macromolecule to which they are bound, two of whose components are labelled S_1 and S_2; and one due to spin labels which have some independent motion, two of whose components are labelled W_1 and W_2. These two conformations of the spin label are referred to as, respectively, the strongly-immobilized and the weakly-immobilized state. Examples of these two types of spectra are shown in Figs. 1B and 1C.

The ratio of the amplitudes of the two peaks W_1 and S_1 was taken as a measure of the relative amounts of the two types of state. Use of this amplitude ratio implies a two state model for the system, a procedure which would be valid only if the properties of the spin label in the two conformational states did not change from one type of experiment to another. Changes in the properties of the weakly-immobilized label can be detected from the ratio of the peaks labelled W_1 and W_2, and in those of the strongly-immobilized label from the width of S_1 and the separation between S_1 and the center of the spectrum[25]. These criteria indicated that the two state model applied when the amplitude ratio was less than 20, with the exception of the spectrum given by *n*-butanol-extracted material while butanol was still present. The changes in individual ESR line widths observed at amplitude ratios greater than 20 were not investigated in detail since this ratio indicates that most of the labelled groups are present in a weakly-immobilized state[25] and hence, the labelled proteins have an extremely disrupted structure which is of little interest in the present study.

Solubilization generally resulted in an increase in amplitude ratio (Table I, Fig. 2), which corresponds to an increase in the proportion of weakly-immobilized spin label. An exception was the *n*-butanol-treated material when butanol was still present. However, this material was also unusual in that its spectra exhibited changes in the state of the weakly-immobilized component, while the amplitude ratio was close to that of the control (Fig. 3).

The insoluble fractions yielded spectra which were either identical to those which were soluble (deoxycholate, NaOH, urea) or had a lower amplitude ratio (HCl), corresponding to a relatively smaller proportion of weakly-immobilized components.

Dialysis of the solubilizer-containing fractions against hypotonic phosphate (pH 7.6) caused their amplitude ratios to decrease (Table II) and resulted in additional fractions for the deoxycholate, NaOH, HCl, and urea-treated samples. The soluble butanol, deoxycholate, and urea samples yielded amplitude ratios close to the value of 4.1 ± 0.1 found with similarly-dialyzed controls, suggesting that the solubilizer-induced structural changes had been largely reversed. The insoluble fractions obtained after dialysis had roughly similar or higher amplitude ratios than the corresponding soluble ones, except for insoluble fractions obtained on dialyzing the material previously treated with HCl. Dialysis of the lysolecithin-treated ghosts caused the amplitude ratio to increase from 6.5 ± 0.2 to 7.3 ± 0.3. However, interpretation of this

result is difficult because the dialysis procedure employed did not remove the lysolecithin completely. At the temperature used, 4°, the lysolecithin precipitated in the dialysis bag, presumably because of a marked decrease in solubility.

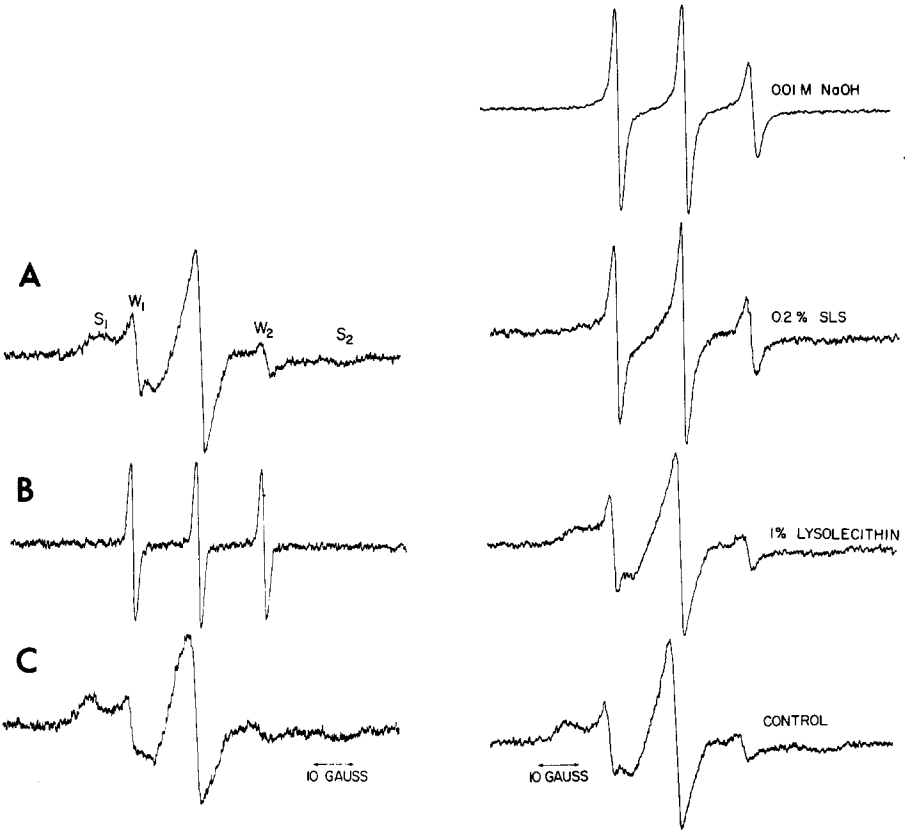

Fig. 1. (A) ESR spectrum of the spin-labelled ghosts in 20 mosM phosphate buffer (pH 7.6). S_1 and S_2 are lines due to strongly-immobilized labels and W_1 and W_2 are due to weakly-immobilized labels. (B) ESR spectrum of free nitroxide maleimide (Mal-6) in hypotonic phosphate (pH 7.6) (all labels moving freely). (C) ESR spectrum of spin-labelled ghosts at pH 4.9 (relatively few weakly immobilized labels present).

Fig. 2. ESR spectra of soluble fractions obtained by various procedures. SLS, sodium lauryl sulfate.

Dialysis of the labelled cells in hypotonic phosphate against the same buffer at 4° for 3 days, or storage at 4° for the same length of time, caused a small increase in the amount of weakly-immobilized component, the amplitude ratio going from 3.8 ± 0.1 to 4.1 ± 0.1. In addition, about 2 % of the protein became soluble. The spectrum of the washed, insoluble material was comparable to that of the original mixture while the soluble fraction had more of the weakly immobilized component. These small changes indicate that the properties of the labelled membrane proteins change slowly with time. However, the change is not large enough to preclude the use of dialyzed ghosts as controls.

The selectivity of Mal-6 for sulfhydryl groups was demonstrated by the marked inhibition (about 99 %) of labelling caused by preincubation for 12 h at 4° with 1 mM

TABLE I

AMPLITUDE RATIO IN PRESENCE OF SOLUBILIZER AND PER CENT PROTEIN SOLUBILIZED

The amplitude ratio of the control refers to the ratio obtained prior to the addition of solubilizer. The value of 5.5 ± 0.1 was obtained when the cells were in water and the value of 3.8 ± 0.1 when they were in hypotonic phosphate (pH 7.6).

Solubilizer	*Amplitude ratio*			*% of protein solubilized*
	Control	*Soluble fraction*	*Insoluble fraction*	
n-Butanol	5.5 ± 0.1	5.1 ± 0.1	⩽5.1*	85 ± 5
1% lysolecithin	3.8 ± 0.1	6.5 ± 0.2		>96
Deoxycholate				
2.5 μmoles/mg protein	3.8 ± 0.1	7.0 ± 0.1	6.9 ± 0.1**	52 ± 4
5.0 μmoles/mg protein	3.8 ± 0.1	8.6 ± 0.1		97.5 ± 2.5
0.2% sodium lauryl sulfate	3.8 ± 0.1	22 ± 3		98
0.01 M HCl	3.8 ± 0.1	80 ± 20	10 ± 1	50 ± 10
0.01 M NaOH	3.8 ± 0.1	∞	∞	52 ± 2
33% pyridine	5.5 ± 0.1	>90		100
8 M urea	3.8 ± 0.1	∞	∞**	72

* Refers to insoluble interfacial material. Insufficient available for more precise estimate of amplitude ratio.

** These fractions were not washed since washing with solubilizer greatly reduced their amount.

TABLE II

AMPLITUDE RATIO AFTER DIALYSIS

Control value of the amplitude ratio is 4.1 ± 0.1. Controls consisted of samples to which solubilizer had not been added, which were then dialyzed under conditions identical to those containing solubilizer.

Solubilizer	*Amplitude ratio*					
	Soluble fraction			*Insoluble fraction*		
	Soluble	*Insoluble*	*% soluble*	*Soluble*	*Insoluble*	*% soluble*
n-Butanol	5.4 ± 0.1					
1% lysolecithin	7.3 ± 0.3					
Deoxycholate						
2.5 μmoles/mg protein	5.2 ± 0.1			4.5 ± 0.1	7.5 ± 0.1	35
5.0 μmoles/mg protein	5.5 ± 0.1					
0.2% sodium lauryl sulfate	15 ± 2					
0.01 M HCl	>90	2.5 ± 0.3	59	>90	20 ± 2	3.7
0.01 M NaOH	>90	>90	88	>90	>90	5
33% pyridine	10 ± 2			10 ± 2		
8 M urea	5.6 ± 0.6	38 ± 4	39	3.9 ± 0.2	25 ± 3	25

N-ethylmaleimide or *p*-chloromercuribenzoate, or 0.1 mM $HgCl_2$, in agreement with SANDBERG AND PIETTE[23]. The labelled ghosts were found to contain 5 nmoles of spin-labelled groups per mg of protein. The spin concentration was determined by dissolution of the labelled ghosts in 0.1 M NaOH to produce a narrow line ESR spectrum

such as Fig. 1B, and comparing the intensity of this spectrum with that of Mal-6 at known concentration. The number of SH groups modified by Mal-6 was computed to be about 10 % of the total detectable by *p*-chloromercuribenzoate titration[18], which is equivalent to labelling not more than 1 amino acid residue per thousand in the ghost proteins. In agreement with others[18], the soluble fraction obtained after butanol

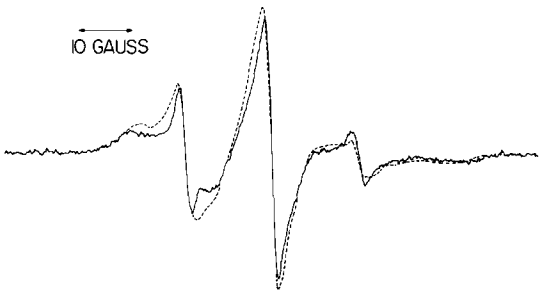

Fig. 3. Effect of *n*-butanol on ESR spectrum. ———, spin labelled ghosts in water; – – – – labelled ghosts after extraction of lipids with *n*-butanol.

extraction contained much less lipid than the original ghosts, the phospholipid and cholesterol contents being approx. 10 % and 1 % of the weight of the proteins, respectively.

DISCUSSION

The present investigation indicates that large differences in spectra can be caused by the different solubilization methods. These spectral changes may be the results of protein conformation changes. Alternately, or in addition, they may be caused by a change in microenvironment of the spin label, possibly resulting from a change in protein–lipid interactions. The precise reasons for the changes in each case are not known. However, the salient point at present is that fractions could be obtained in several instances after removal of solubilizing agent whose spectra resembled that of the untreated, labelled ghosts. With some solubilization procedures such reversal occurred only in certain fractions, for example, with two out of four and one out of three following urea and HCl treatment, respectively (Table II). These reversal results suggest that the structures of the labelled proteins were similar to those when they were part of a membrane, certainly insofar as the labelled site is concerned. Support for this view is provided by the results of other solubilizaton studies. Dissolution using *n*-butanol and sodium deoxycholate produced amplitude ratios close to the control values while allowing retention of some enzyme activities[18,26]. In contrast, solubilizers causing large changes in amplitude ratio, (urea, lysolecithin, extremes of pH) resulted in changes in optical rotatory dispersion behavior[27] as well as loss of enzymic and immunochemical activities[14]. The significance of the reversible ESR spectral changes is the provision of an additional criterion for judging which solubilization methods, or which fractions yielded by a particular solubilization method, are of greater interest in subsequent fractionation studies whose aim is the isolation of proteins retaining structural integrity.

The use of the spin label method for detecting conformational changes in com-

plex protein systems such as membranes has two advantages over other physical techniques (optical rotatory dispersion, circular dichroism, ultraviolet difference spectroscopy) which are worth emphasizing. One is that information about structural changes can be obtained with insoluble as well as soluble materials. It is noteworthy in this connection that two of the fractions with amplitude ratios close to the control (HCl and sodium deoxycholate, Table II) were insoluble. The other advantage is that it permits the focussing of attention on only the labelled proteins. This means that information about structural changes in the labelled protein(s) may be obtainable without regard to what has happened to other macromolecular components. This information may be obtained after the labelled proteins have been isolated and purified, or when only partially purified, as may be the case with some of the fractions obtained in the present study. Labels are now available for a variety of amino acids, so different regions of a particular protein may be monitored[10,25].

A complication may arise in isolation studies where more than one protein is labelled and the ESR spectra of these proteins differ. To compare the spectra of the isolated labelled proteins with that of the labelled membranes an appropriate summation procedure must then be carried out. The number of proteins spin-labelled in the present study is not known. However, it turned out that summation procedures were unnecessary with one exception—the fractions obtained from the HCl soluble portion of labelled ghosts after dialysis. It was found that the insoluble fraction had an amplitude ratio (2.5 ± 0.2) lower than the control (4.1 ± 0.1) while the corresponding soluble one had a higher value (>90). This raised the possibility that selective solubilization had taken place of those proteins which in the membrane have a higher proportion of weakly-immobilized groups. However, summation of the spectra, taking into account the relative amounts of the two fractions, yielded an amplitude ratio of approx. 30. This suggested that if selective solubilization did occur it was also accompanied by a change in conformation in the protein(s) of one, or both, of the fractions.

In addition to providing information about structural changes on dissolution, the results have also been useful in two other ways. (1) They imply, in agreement with suggestions provided by other studies[28,29], that the structures of some of the membrane proteins are not highly dependent on the presence of lipids (the *n*-butanol-solubilized material was relatively free of lipid while its ESR spectrum was similar to that of the labelled ghosts). A novel aspect of the spin label experiments in this connection is that the free radical may provide a marker to aid in the eventual identification of such proteins in human erythrocyte ghosts. (2) The results provide suggestions as to the mechanism by which the various solubilizers work. On the one hand relatively small changes in amplitude ratio were caused by the reagents which can interact with lipids — sodium deoxycholate, lysolecithin, *n*-butanol. On the other hand, appreciably larger changes were caused by urea, pyridine, and extremes of pH — conditions which can denature proteins. This behavior is consistent with solubilization occurring with the first group because of their interactions with lipids, while with the second group, because of their disruptive effect on the structure of the proteins. The position of sodium lauryl sulfate in the comparison is difficult to define because it can interact with lipids as well as cause changes in the conformation of proteins[30,31].

CONCLUSION

The potential of the spin label method for detecting structural changes in membrane proteins on dissolution has been investigated. The fact that fractions were obtained with minimal spectral changes were observed suggests that the method may be useful in the isolation of membrane proteins with retention of structural integrity, particularly for those which do not contain obvious self-markers sensitive to conformation perturbations. The results have also been useful in comparing environmental changes of the labelling group caused by different dissolution procedures, in indicating the proteins whose structures are not markedly lipid-dependent, and in probing the mechanism of action of the solubilizers employed. Some advantages and complications of the method were discussed.

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