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Effect of proteolysis on the electron spin resonance spectra of maleimide spin labeled erythrocyte membrane

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The ratio of low-field amplitudes of weakly and strongly immobilized signals of ESR spectra of a maleimide spin label bound to erythrocyte membranes (h_w/h_s) increases progressively during incubation at 37°C. This increase is due to the 'self-digestion' of membrane proteins by endogenous proteinases and is attenuated by proteinase inhibitors. Digestion of membranes with chymotrypsin also increases the h_w/h_s ratio. These results suggest a need for a careful interpretation of data from spin-labeled membrane proteins, especially in experiments involving prolonged incubations of membrane preparations when the proteolytic effects may be significant.

Introduction

Analysis of ESR spectra of cellular membranes spin-labeled with maleimide nitroxide derivatives has become an acknowledged methodology in studies of membrane proteins. Though information derived in this way is rather nonspecific, the spectra are very sensitive to changes in the physical state of membrane proteins, so the method has been widely used in studies of effects of various physical and chemical factors on membranes, and of membrane protein alterations under pathological conditions (e.g. Refs. 1–4). The ESR spectra of membrane-bound maleimide spin labels are usually complex and are analysed in terms of the ratio of low-field signal amplitudes of weakly and strongly immobilized, respectively, spin-label residues (h_w/h_s) (Fig. 1). This ratio has been reported to depend on such parameters as pH, ionic strength and composition of the medium, temperature and time of labeling [5,6]. It has been reported [5] that incubation of erythrocyte membranes at 37°C results in an irreversible increase of the h_w/h_s ratio of membrane-bound maleimide spin label. This prompted us to suggest that the list of usually considered determinants of this ratio is not complete. Incubation of erythrocyte membranes at this temperature involves a 'self-digestion' of membrane proteins [7,8]. In this study, evidence is presented demonstrating that degradation of erythrocyte membrane proteins by endogenous or exogenous proteinases brings about an increase in the h_w/h_s ratio of a membrane-bound maleimide spin label.

Material and Methods

Maleimide spin label, *N*-(1-oxyl-2,2,6,6-tetramethylpiperid-4-yl)maleimide (Mal-6) was purchased from Reanal (Hungary). Bovine blood was

Abbreviations: ESR, electron spin resonance; Mal-6, *N*-(1-oxyl-2,2,6,6-tetramethylpiperid-4-yl)maleimide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

obtained in a local abbatoir. Erythrocyte membranes were prepared by hypotonic hemolysis in 20 mM sodium phosphate (pH 8.0) and successive washings with 20, 10 and 5 mM phosphate (pH 8.0). The membranes (3 mg protein/ml, in 5 mM phosphate) were spin-labeled by incubation with 0.5 mM Mal-6 for 1 h at room temperature. Unbound label was removed by washing four or five times with the same buffer. Except for incubation with the label, the procedures of membrane preparation and washing were performed on ice, at a temperature of 0–3°C. Unless stated otherwise, spin-labeled membranes (5 mg protein/ml) were incubated in 5 mM phosphate (pH 8.0) at 37°C, in the presence of 100 μg/ml chloramphenicol (Serva) to prevent bacterial growth. ESR spectra were taken in a SE/X-28 spectrometer (Wrocław Technical University, Poland) at ambient temperature (18°C). Electrophoresis of membrane proteins was performed according to Laemmli [9]. The electrophoretograms were stained with Coomassie brilliant blue R. Appropriate protein bands were quantitated by cutting out gel slices, extraction of the dye with 0.1 M NaOH/0.2% SDS and measurement of absorbance at 600 nm.

Results and Discussion

Under the experimental conditions employed, freshly isolated bovine erythrocyte membranes labeled with Mal-6 yielded a h_w/h_s ratio (Fig. 1) of 2.35 ± 0.38 (mean ± S.D., $n = 8$). Incubation of spin-labeled membranes at 37°C brought about a progressive increase in the h_w/h_s ratio (Table I). Simultaneously, 'self-digestion' of membrane proteins took place, detectable by a decrease in the intensity of high-molecular-weight protein bands

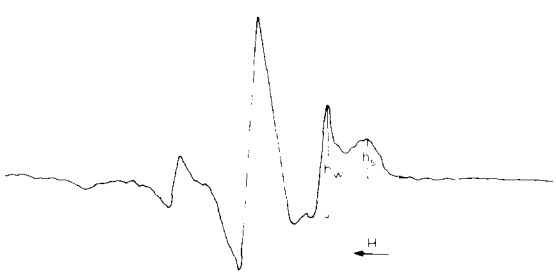

Fig. 1. ESR spectrum of Mal-6 bound to bovine erythrocyte membranes.

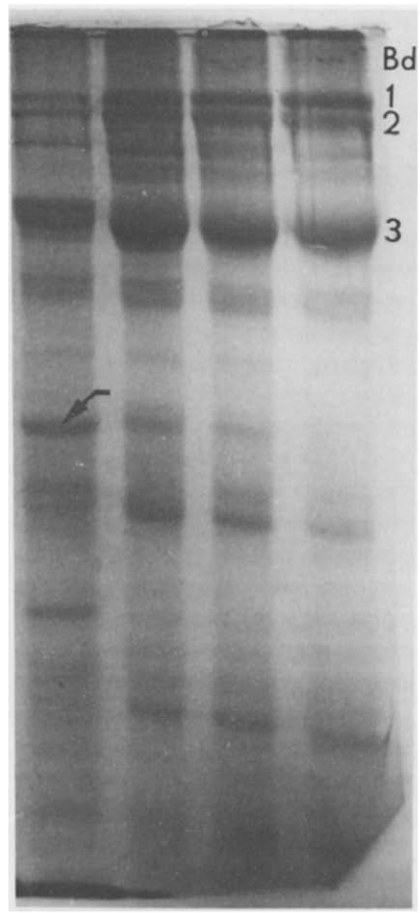

Fig. 2. Electrophoretograms of freeze-thawed Mal-6-labeled erythrocyte membranes incubated for 65 h at 37°C. From left to right: no inhibitor, PMSF, EDTA and PMSF + EDTA. The arrow points to the proteolytic product of apparent molecular weight of 51 000.

(especially spectrin and band 3) and an increase in the amount of low-molecular-weight proteins (Fig. 2, Table I). Both these effects might represent only parallel phenomena, but several lines of evidence suggest a causal relationship between them:

(i) When erythrocyte membranes were incubated in the presence or proteinase inhibitors (PMSF, EDTA or both) the rate of increase in the h_w/h_s ratio was attenuated, in parallel with inhibition of proteolysis, as judged from the extent of disappearance of spectrin and band 3, and of the increase in the amount of a degradation product of apparent molecular weight of 51 000 (Fig. 2, Table I).

TABLE I

EFFECT OF PROTEINASE INHIBITORS ON THE h_w/h_s RATIO OF Mal-6 BOUND TO ERYTHROCYTE MEMBRANES AND ON THE RELATIVE PROTEIN BAND CONTENT AFTER INCUBATION AT 37°C

Mean values from three experiments; PMSF, 20 μg/ml; EDTA, 1 mM.

	No inhibitor	PMSF	EDTA	PMSF + EDTA
h_w/h_s				
0 h	2.19	2.19	2.19	2.19
26 h	3.69	3.07	2.42	2.09
47 h	4.92	3.76	2.93	2.32
65 h	8.83	3.89	3.39	2.42
Relative protein band content after 65 h (%)				
Spectrin (bands 1 + 2)	100	208	219	246
Band 3	100	154	139	180
Product (51 kDa)	100	102	65	51

(ii) When membrane preparations from three animals were compared, the rate of increase in the h_w/h_s ratio correlated with the rate of degradation of membrane proteins (Table II).

(iii) Action of chymotrypsin on the spin-labeled membranes induced a rapid increase in the h_w/h_s ratio, faster at a higher enzyme concentration (Fig. 3). This digestion resulted in a liberation of some spin-labeled protein fragments from the membrane, since supernatants obtained by centrifugation of membrane suspensions yielded a simple spectrum of weakly immobilized Mal-6 (Fig. 4) while the spectrum of whole suspension remained composite. The spectrum shown in Fig. 4 is not typical of free Mal-6 residue, thus speaking against a possibility or hydrolysis of the label as an alternative source of spectral changes.

(iv) Incubation of membranes in 150 mM rather than 5 mM phosphate considerably slowed down both the increase in the h_w/h_s ratio and the membrane protein 'self-digestion' (not shown).

(v) The ESR spectral changes are not due to a release of spin-label molecules which might have been bound non-covalently to the membranes. Under the washing conditions employed, the h_w/h_s ratio was constant starting from the 3rd wash, i.e. further washing did not remove any label from the membranes. Furthermore, when membranes and eventual soluble degradation products were precipitated with 5% (final concentration) trichloroacetic acid, no spin label was found in the supernatants, either before or after 48-h incubation.

Similar spectral effects (increase in the h_w/h_s ratio) of Mal-6-labeled membranes upon incubation at 37°C were also observed with human, rabbit and hamster erythrocyte membranes.

TABLE II

CHANGES IN THE h_w/h_s RATIO OF Mal-6 BOUND TO ERYTHROCYTE MEMBRANES AND RELATIVE CONTENT OF MAIN PROTEIN BANDS OF MEMBRANES FROM DIFFERENT ANIMALS AFTER 16-h INCUBATION

	Fresh membranes			Freeze-thawed membranes		
Animal No.	1	2	3	1	2	3
h_w/h_s						
0 h	2.41	2.16	3.16	2.30	2.08	3.00
16 h	12.72	15.73	7.23	6.80	6.22	5.21
Protein content ratio 16 h/0 h (%)						
Bands 1 + 2	34	31	62	61	76	88
Band 3	31	76	94	66	96	83

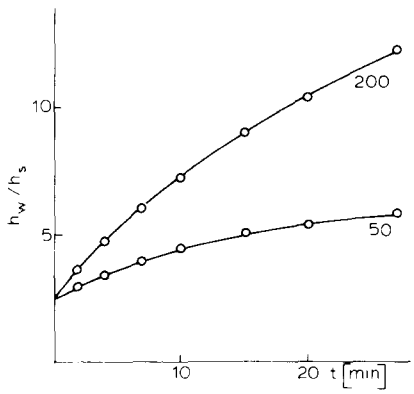

Fig. 3. Time-course of changes in the h_w/h_s ratio of Mal-6-labeled erythrocyte membranes incubated with chymotrypsin (membranes: 5 mg protein/ml, chymotrypsin (Miles): 50 and 200 μg/ml; temperature: 18°C).

Freezing the bovine erythrocyte membranes at −20°C and subsequent thawing did not induce significant alterations in the h_w/h_s ratio but slowed down both the 'self-digestion' and the ESR spectral changes (Table II).

These results demonstrate that proteolytic degradation of erythrocyte membrane proteins induces changes in the conformation and/or disposition of Mal-6-binding fragments of protein molecules which result in an increase of the fraction of weakly immobilized spin-label residues. It is difficult to judge from these data whether that effect is a general feature of all membrane proteins or a contribution of some protein fraction(s) is dominating. Both the proteolysis and the increase in the h_w/h_s ratio proceeded in whole membranes, in membranes extracted with phosphate-buffered physiological saline (in order to remove band 6 protein), in membranes extracted with 0.1 mM EDTA (pH 8.5) (to remove spectrin) and in membranes extracted subsequently with 1 M KCl/25 mM EDTA (pH 6.75) (to elute band 2.1 and band 4.1 proteins). It was hardly possible to study membranes stripped with 0.1 M NaOH (to elute all peripheral proteins) because of an enormous rise in the h_w/h_s ratio induced by the procedure itself. However, we observed also an increase in the h_w/h_s ratio of such proteins as bovine serum albumin and hemoglobin labeled with Mal-6 and subjected to the action of proteolytic enzymes (Grzelińska and Bartosz, unpublished data). It suggests that this effect is a general phenomenon of proteolytic unfolding of protein structure leading to a release of bound Mal-6 residues from interactions restricting their mobility.

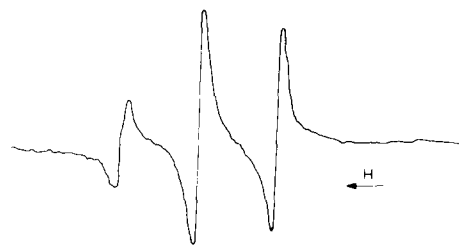

Fig. 4. ESR spectrum of supernatant from chymotrypsin-digested Mal-6-labeled erythrocyte membranes (200 μg chymotrypsin/ml; 1h).

Spin-labeling with Mal-6 did not inhibit the 'self-digestion' of erythrocyte membrane proteins (not shown).

The results obtained point to: (i) The necessity of a careful interpretation of studies of spin-labeled membrane proteins, especially in cases of prolonged incubations at ambient and elevated temperatures; (ii) The need of employment of proteinase inhibitors in such studies.

It seems possible that progressive proteolysis in situ may at least contribute to the increase in the h_w/h_s ratio of Mal-6-labeled erythrocyte membranes during red cell aging [10].

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