CONFORMATIONAL CHANGES OF SPIN-LABELED MEMBRANE PROTEINS IN HUMAN ERYTHROCYTES

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1. Introduction

The spin label method has been successfully applied to investigations of the dynamic behavior and function of biological membranes [1–3]. The membrane fluidity, as an important factor of the dynamic properties, has been used to study the membrane organization in normal or abnormal cells [4,5]. To analyse a response of the membrane fluidity due to external perturbations, it is important to understand the behavior of the membrane proteins as well as lipids. Electron spin resonance (ESR) technique is the most useful one to monitor sensitively the molecular motion over 10^{-11}–10^{-3} s [6].

The maleimide-analogue sulfhydryl spin labels allow us to study the conformational changes of membrane proteins [7–9]. The ESR spectra of erythrocyte ghosts labeled with maleimide have shown the simultaneous existence of strongly and weakly immobilized components. The ratio of the signal amplitude of both components has been used as an index of protein conformational changes [8,9], but varies significantly under several conditions. It is therefore worthwhile to give a criterion for the factors which influence this ratio. We show here how this ratio in erythrocyte ghosts is affected by the label concentration, temperature, pH, cholesterol content and ghost preparation.

2. Materials and methods

Erythrocyte ghosts were prepared from human red blood cells of healthy adult donors by the methods in [10]. White ghosts were resealed by incubating in 10 mM sodium phosphate buffer (pH 7.3) containing 0.15 M NaCl (PBS) for 1 h at 37°C [11].

To label the membrane proteins with 4-maleimide-2,2,6,6-tetramethylpiperidinooxyl (Syva Co.), 20 μl 4 mM label solution dissolved in hexane into a sample tube was evaporated in vacuo for a few minutes, to which 2 ml ghost suspension (0.1 mg protein/ml) in PBS (pH 7.3) was added. After incubation for 2 h at 0°C, ghosts were washed with the chilled buffer to remove free spin labels. Protein concentrations were determined as in [12].

The effect of pH on the conformation of membrane proteins was studied in the following buffers: pH 3.0–4.9, 0.15 M NaCl, 10 mM sodium acetate; pH 5.4–7.3, 0.15 M NaCl, 10 mM sodium phosphate; pH 7.8–9.1, 0.15 M NaCl, 10 mM Tris; pH 10.0–11.0, 0.15 M NaCl, 10 mM glycine. Sample was incubated in each buffer for 30 min at 0°C, centrifuged and packed into a hematocrit capillary tube.

Cholesterol-enriched or -depleted erythrocyte ghosts which mediated the cholesterol content as in [13] or by incubating normal ghosts with cholesterol-modified dipalmitoylphosphatidylcholine liposomes. Cholesterol was determined as in [14] and phosphate as in [15].

ESR spectra were recorded on a JEOL JES FE-1X spectrometer equipped with a variable temperature accessary.

3. Results and discussion

The ESR spectra of maleimide-labeled ghosts indicated strongly (s) and weakly (w) immobilized components which may be given by the label molecules

Abbreviations: ESR, electron spin resonance; PBS, phosphate-buffered saline; C/P, cholsterol: phospholipid molar ratio

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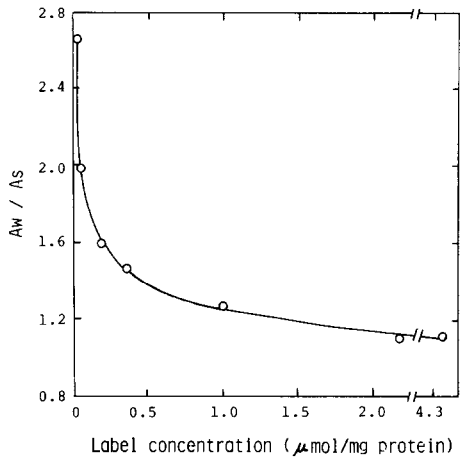

Fig.1. Effects of label concentration on the A_w/A_s ratio of maleimide-labeled ghosts. Ghosts were incubated with various concentrations of the spin label indicated above at 0°C for 2 h in PBS (pH 7.3). The spectra were recorded at 25°C.

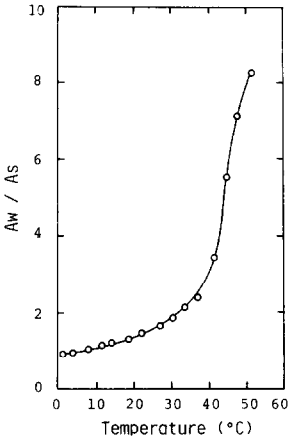

Fig.2. Effects of temperature on the A_w/A_s ratio of maleimide-labeled ghosts. Ghosts were incubated with 0.3 μmol label/mg protein at 0°C for 2 h in PBS (pH 7.3).

bound to the sulfhydryl groups, buried within the membrane protein matrix and exposed at the surface of the proteins, respectively. The amplitudes, A_s and A_w, of the 's' and 'w' signals were measured vertically from the horizontal base line to each peak at the low field as in [9]. The A_w/A_s ratio has been used as an index of protein conformational changes, i.e., the increase in its ratio represents the unfolding of proteins.

3.1. *Effect of label concentration*

Fig.1 shows a relationship between the A_w/A_s ratio and the amount of label added to membrane proteins. The increase of label concentration demonstrated the decrease in the A_w/A_s values. The ratio changed significantly with <0.2 μmol label/mg protein. This may indicate that the label molecules first react with the sulfhydryl groups exposed on the membrane proteins and then with the buried ones.

3.2. *Temperature effect*

Fig.2 indicates the temperature dependence of the A_w/A_s ratio in ghosts. This ratio increased gradually over 2–30°C but rose abruptly at >30°C. Similar behavior has been observed in spectrin extracted from human erythrocytes [16]. The reversibility in the dependence upon temperature of the A_w/A_s ratio was studied as follows. The ghosts measured in PBS (pH 7.3) at 25°C were preincubated at any settled temperature for 30 min, then for 2 h at 0°C and their ESR spectra were recorded at 25°C. The A_w/A_s values changed reversibly at ≤40°C. At higher temperatures, the A_w/A_s values in preincubated ghosts were higher than those in the native samples.

3.3. *pH effect*

The effect of pH on the conformation of membrane proteins was investigated over pH 3.0–11.0 (fig.3). The A_w/A_s values were small and constant at pH < 4.5, then increased gradually with rising pH except for the marked increase at pH > 8.0. The

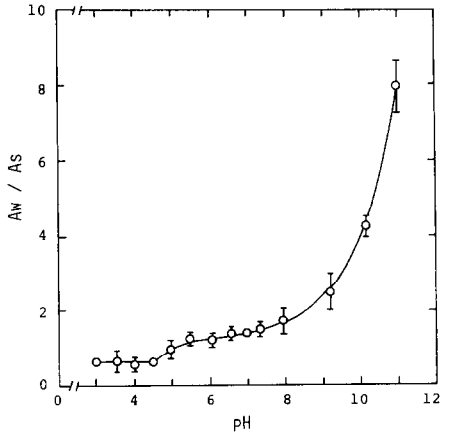

Fig.3. Effects of pH on the A_w/A_s ratio of maleimide-labeled ghosts. Ghosts labeled as in fig.2 were incubated in each buffer for 30 min at 0°C. The spectra were recorded at 25°C. Each point represents the mean of 3 expt ± SEM.

reversibility in the dependence upon pH of the A_w/A_s value was studied as follows. The spectrum of the spin-labeled ghosts in pH 7.3 buffer was measured at 25°C. The sample was allowed to stand for 30 min at 0°C in each buffer indicated in fig.3, then incubated in pH 7.3 buffer for 2 h at 0°C and its ESR spectrum was recorded. The reversiblity was complete over pH 3.0–9.1 but was only partial at pH > 10.0. The decrease of the A_w/A_s ratio at low pH may indicate the reversible folding or aggregation of membrane proteins [17–19].

The temperature dependence of the A_w/A_s ratio was predominantly controlled by pH. Over 2–40°C, the A_w/A_s values in pH 9.1-treated ghosts increased from 2.0–8.0, while those in pH 5.5 changed from 0.8–2.3. This indicates that the membrane proteins at low pH are folded or aggregated and resist changes in temperature.

3.4. *Cholesterol effect*

When the level of cholesterol in membranes is modified, it has been discussed about whether vertical displacement or conformational change of membrane proteins in lipid bilayers occurs [20,21]. We have used maleimide label to study the effect of cholesterol on the conformational changes of membrane proteins. The temperature dependence of the A_w/A_s ratio in ghosts with various cholesterol contents (C/P = 0.7 and 1.8) was the same as that in normal ghosts (C/P = 1.0) over 2–50°C, indicating that the conformational changes of membrane proteins for temperature are unaffected by the content of cholesterol examined here. This result is consistent with that obtained from the paramagnetic fluorescence quenching which suggests the existence in clusters of cholesterol segregated away from proteins [22,23].

3.5. *Membrane preparation*

The A_w/A_s value was also influenced by the ghost preparation. The spectrum of unsealed ghosts labeled with maleimide in 5 mM phosphate buffer (pH 7.3) gave the A_w/A_s value of 2.0 at 16°C, which is larger than that (A_w/A_s = 1.5) in resealed ghost in PBS (pH 7.3).

The present studies indicate that the A_w/A_s value in ghost is significantly affected by label concentration, temperature, pH and ghost preparation, but not by cholesterol content. The conformational changes of membrane proteins are predominant at >30°C and pH > 8, and irreversible at >40°C and pH > 10.

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