MOLECULAR DYNAMICS OF SPECTRIN–ACTIN AT LOW pH

Saturation transfer EPR studies

L. W.-M. FUNG, M. J. SOO HOO and W. A. MEENA
Department of Chemistry, Wayne State University, Detroit, MI 48202, USA

Received 23 July 1979

1. Introduction

The significance of the peripheral erythrocyte membrane proteins, of which spectrin is the principle component, in maintaining the shape of the red blood cells, has recently been recognized [1,2]. One approach to study the cell shape and deformability is to monitor the perturbation response of membrane components. Many membrane perturbations leading to the formation of aggregates have been associated with the protein components, particularly with spectrin–actin precipitation [3,4]. We have studied the dynamics of the membrane proteins in pH-induced aggregation [5] by spin label electron paramagnetic resonance (EPR) techniques. The sulfhydryl groups of both intact erythrocyte membranes and the isolated spectrin–actin complex* at pH 8 and pH 4.5 have been labelled with *N*-(1-oxyl-2,2,6,6-tetramethyl 4-piperidinyl)maleimide (Mal-6). Conventional EPR and saturation transfer (ST) EPR spectra at various temperatures have been obtained. ST EPR spectroscopy is a useful technique for monitoring slow rotational motion (with correlation times $>10^{-7}$ s [6,7]), and has been used to study several proteins in membrane systems [8,9].

Our results demonstrate the strong sensitivity of the ST EPR method in detecting pH induced perturbation in erythrocyte membranes at different temperatures. In addition, our results show that, under equivalent conditions, the low pH spectrin–actin complex exhibits motional behavior which is both qualitatively and quantitatively similar to that of the membrane-associated complex. These results indicate that the spectrin–actin complex is only weakly associated with other membrane components, in agreement with the fact that these peripheral proteins can be extracted easily from the membrane matrix. The molecular motions of the labelled spectrin–actin appear to be unrestricted by other membrane components. Recently considerable progress has been made in purifying membrane proteins and in the understanding of the structure of spectrin and the interaction between spectrin and some specific membrane components [10–12]. The strong sensitivity of the ST EPR technique to molecular motions in erythrocyte membranes indicates that this is a useful and probably unique method for quantitatively studying the dynamics of these systems.

2. Materials and methods

Human red blood cells were obtained from the Detroit Red Cross Blood Bank and used within 1 week after drawing. Hemoglobin-free white erythrocyte membrane ghosts were obtained by the methods in [13,14]. The cells were washed with 150 mM NaCl saline solution buffered with 5 mM sodium phosphate at pH 8 (PBS), and were lysed and washed in 5 mM phosphate buffer at pH 8 (5P8). Spectrin–actin was prepared by the methods in

* The term spectrin–actin is used here to represent those peripheral proteins which are easily extracted from the membranes, with bands I and II as the major proteins in their electrophoresis gels (see section 2)

[15]. White membrane ghosts were incubated at 37°C in low ionic strength buffer (0.3 mM phosphate buffer at pH 7.6) to solubilize spectrin–actin; the insoluble components were removed by ultracentrifugation. Gel electrophoresis was performed on the samples to ensure their purity. Low pH samples of isolated spectrin–actin and of membranes were both prepared by adjusting the pH of the samples to pH 4.5 with HCl. Membrane samples and some spectrin–actin samples at pH 4.5 were centrifuged at 21 000 × *g* for 10 min and the precipitate was then resuspended in 5 mM sodium acetate buffer at pH 4.5. Spectrin–actin at pH 4.5 were also collected either by gravitational sedimentation or by low-speed centrifugation at ~200 × *g*. Mal-6 (Syva) at 30–50 μg/mg protein was used and the sample stirred gently for 1 h in the dark. Excess spin label was removed by washing (usually 4 times) with acetate buffer. EPR signals were recorded after each washing to ensure total removal of the unreacted label. All preparative procedures were carried out at 4°C.

EPR and ST EPR signals were observed on a Varian E109E X-Band Spectrometer with an E238 cavity and a variable temperature accessory. Signals were time averaged when necessary on a Nicolet 535 time averager. First harmonic conventional EPR spectra were recorded at 1 G modulation (100 kHz); ST EPR spectra were recorded at 5 G modulation (50 kHz). Rotational correlation times (τ values) were obtained by the methods in [6].

3. Results

3.1. *Conventional EPR spectra*

Our conventional EPR studies of spin-labelled membranes reveal nitroxide spectra with overlapping weakly and strongly immobilized signals (not shown), similar to those reported [16,17]. The integrated intensity of the minor component, the weakly immobilized signal, is usually ~10% of the total intensity for membrane samples at pH 8 and room temperature. Upon lowering the pH values and/or temperatures of the samples, the amplitude of the weakly immobilized signal decreases gradually. At pH 4.5, the minor component disappears almost completely. However the hyperfine splitting, and the half-height full-widths of the low/high field components of the major signal, the strongly immobilized signal,

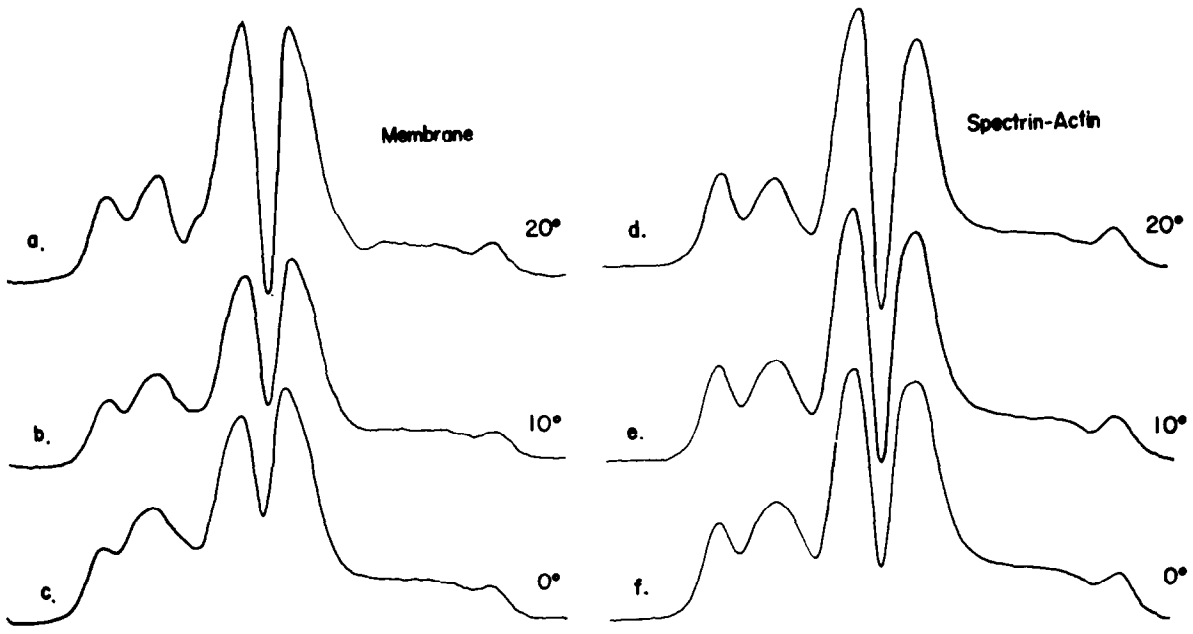

Fig.1. Saturation transfer EPR spectra of (a–c) membrane and (d–f) spectrin–actin aggregates at pH 4.5 at 20°C, 10°C and 0°C.

are insensitive to pH and temperature variations and remain essentially unchanged.

3.2. *Saturation transfer EPR spectra*

Figure 1 shows the 50 kHz modulated ST EPR spectra of the Mal-6-labelled membranes and of the isolated spectrin–actin complex at various temperatures. The spectrin–actin complex is collected by low-speed (200 × *g*) centrifugation. Both series of spectra show substantial sensitivity to temperature change. Membrane samples (spectra a–c) and isolated spectrin–actin samples (spectra d–f) at pH 4.5 give similar, but not identical spectra at each temperature (0°C, 10°C, 20°C). Figure 2 shows the spectra of two low pH spectrin–actin samples as pelleted by high-speed (21 000 × *g*) (spectrum a) as well as by low-speed (200 × *g* or less) (spectrum b) centrifugation. Spectrum a, the sample pelleted at high speed, exhibits comparatively much slower motion than spectrum b. Spectrin–actin or membrane samples can be resolubilized by adjusting the sample back to pH 8 and a spectrum of faster motion reappears.

The ST EPR spectral parameters, L,L'',C,C',H and H'', introduced in [6] were measured. The ratios, L''/L, C'/C and H''/H, are presented in table 1.

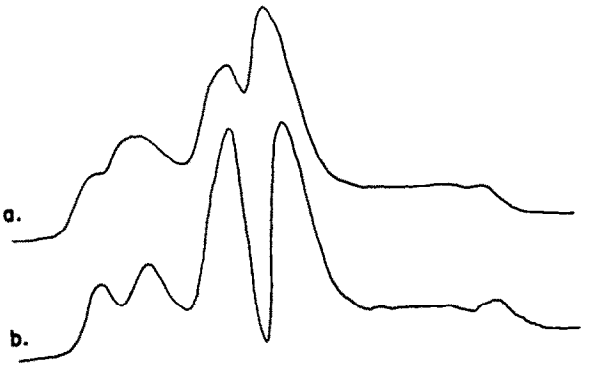

Fig.2. Saturation transfer EPR spectra of spectrin–actin aggregates collected: (a) by high-speed centrifugation; (b) by low-speed centrifugation at 10°C.

4. Discussion

Erythrocyte membranes contain several kinds of proteins, with band III and spectrin–actin being the major components [18]. Spin labelling all of the membrane proteins by Mal-6 might be assumed to give a very heterogeneous system of labelled sulfhydryl groups. However, our studies indicate that >80% of the signal intensity arises from labelled sulfhydryl groups on the cytoplasmic (inner) surface of the membranes, and that ⩾70% of the total signal intensity arises from the spectrin–actin complex (L.F., M. Simpson, in preparation). Thus, the spin label spectra can be used qualitatively to monitor the molecular properties of the spectrin–actin complex in intact membranes.

The success of using the spin labelling technique to study protein dynamics also depends on the lack of residual motion of the spin labels. Since the ST EPR techniques extend the sensitivity to slow motion, the restriction on residual motion of the spin labels is also extended. Although it is difficult to directly

Table 1
Rotational correlation times of erythrocyte membranes under different experimental conditions

System	Temp. (°C)	L''/L	τ_L (s)	C'/C	τ_C (s)	H''/H	τ_H (s)
Membrane	20	1.27	$>10^{-3}$	−0.07	5×10^{-6}	0.92	7×10^{-4}
pH 4.5	10	1.40	$>10^{-3}$	0.31	2×10^{-5}	1.11	$>10^{-3}$
(fig.1a–c)	0	1.50	$>10^{-3}$	0.51	2×10^{-4}	1.23	$>10^{-3}$
Spectrin–actin	20	0.95	4×10^{-4}	−0.17	4×10^{-6}	0.89	6×10^{-4}
pH 4.5	10	1.06	10^{-3}	−0.01	7×10^{-6}	0.97	10^{-3}
(fig.1d–f)	0	1.21	$>10^{-3}$	0.21	1×10^{-5}	1.09	$>10^{-3}$

Parameters L,L'',C,C' H and H'' from [6] are used

demonstrate that the spin labels are totally immobilized within their binding sites, the spectra shown in fig.2 indicate that independent label motion is not a significant problem in this system. The correlation time parameters obtained from fig.2a give τ values longer than the 'totally immobilized' systems used [6]. Furthermore, the decrease in molecular motion of both membrane and spectrin–actin samples under different experimental conditions, as monitored by ST EPR, correlates well with other published ultrastructural properties of these samples under similar conditions. It has been shown that isolated spectrin–actin molecules at neutral pH form large stacked assemblies (hollow cylinders) when the sample is obtained from high-speed centrifugation [19]. Our ST EPR data show that the spectrin–actin complex at pH 4.5 after high-speed centrifugation undergoes very slow motion ($>10^{-3}$ s). Such slow motion would agree well with a large molecular assembly similar to the hollow cylinders. However, if the sample is sedimented by gravity or low-speed centrifugation, the spectra show comparatively faster motion. This again correlates well with the smaller non-stacked assembly observed [19]. Furthermore, the spectral features of fast or slow motion are reversible and are in good agreement with the solubilized (pH 8) or insolubilized (pH 4.5) state of the membranes (data not shown).

Comparing the spectra of membrane with those of spectrin–actin we see very similar, although not quite identical results. The close resemblance between the two systems is direct evidence that the spectrin–actin complex plays an important role in the structural response of erythrocyte membranes to external perturbations. Likewise, the close spectral similarity of the two systems indicates that the motional change of spectrin–actin is virtually independent of protein association with the lipid bilayer and with other membrane components. The small differences observed here may result from membrane components which exist in the membrane sample, but not in the spectrin–actin sample. A more detailed quantitative interpretation will probably require theoretical spectral simulation and/or model system studies. However, direct spectral comparison allows us to detect similarities and differences among systems of interest without resort to theory.

It should also be noted that the existence of protein motion in erythrocyte membranes does not necessarily indicate that the proteins are free to exhibit full rotational or translational diffusion within the membrane. It indicates that the spectrin–actin molecules are able to exhibit a large amplitude angular oscillation (wobbling) or flexing within the membrane network. These motions are pH sensitive. This change in motion certainly indicates a change in the structure of the peripheral protein network and indirectly suggests a change in conformation of the protein molecules. The motions appear to be anisotropic, due to different correlation times, τ_L, τ_C, τ_H, obtained from the same spectrum as indicated in table 1. τ_C is usually faster than τ_L and τ_H in anisotropic motion [7].

In summary, the ST EPR studies of Mal-6-labelled erythrocyte membranes provide unique information of protein dynamics and allow us to quantitatively describe various molecular motions of the spectrin–actin complex in intact membranes and in isolated form at different temperatures. The complex at low pH appears to exhibit anisotropic motion with the different characteristic spectral correlation times ranging from microseconds to milliseconds. These results also indicate that the motional behavior of spectrin–actin at low pH is relatively independent of the lipid bilayer and other membrane components.

Acknowlegements

This work was supported by a Cottrell Research Grant from Research Corporation and research grants from Michigan Heart Association and National Institute of Health (HL 22432).

References

[1] Hainfeld, J. F. and Steck, T. L. (1977) J. Supramol. Struct. 6, 301–311.
[2] Pinder, J. C., Ungewickell, E., Bray, D. and Gratzer, W. B. (1978) J. Supramol. Struct. 8, 439–445.
[3] Kirkpatrick, F. H. (1976) Biochem. Biophys. Res. Commun. 69, 225–229.
[4] Siefring, G. E., Apostol, A. B., Velasco, P. T. and Lorand, L. (1978) Biochemistry 17, 2598–2604.
[5] Gratzer, W. B. and Beaver, G. H. (1975) Eur. J. Biochem. 58, 403–409.

[6] Thomas, D. D., Dalton, L. R. and Hyde, J. S. (1976) J. Chem. Phys. 65, 3006–3024.
[7] Hyde, J. S. (1978) in: Methods in Enzymology. Enzyme Structure (Hirs, C. H. W. and Timasheff, S. N. eds) pp. 480–511, Academic Press, New York.
[8] Kusumi, A., Ohnishi, S., Ito, T. and Yoshizawa, T. (1978) Biochim. Biophys. Acta 507, 539–543.
[9] Baroin, A., Bienuenue, A. and Devaux, P. F. (1979) Biochemistry 18, 1151–1155.
[10] Ralston, G. B. (1978) J. Supramol. Struct. 8, 361–373.
[11] Cohen, C. M., Jackson, P. L. and Branton, D. (1978) J. Supramol. Struct. 9, 113–124.
[12] Goodman, S. R. and Branton, D. (1978) J. Supramol. Struct. 8, 455–463.
[13] Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130.
[14] Steck, T. L. and Kant, J. A. (1974) in: Methods in Enzymology. Biomembranes (Fleischer, S. and Packer, L. eds) pp. 172–180, Academic Press, New York.
[15] Bennett, V. and Branton, D. (1977) J. Biol. Chem. 252, 2753–2763.
[16] Schneider, H. and Smith, I. C. P. (1970) Biochim. Biophys. Acta 219, 73–80.
[17] Butterfield, D. A. (1977) Acc. Chem. Res. 10, 111–116.
[18] Steck, T. L. (1974) J. Cell Biol. 62, 1–18.
[19] Harris, J. R. (1969) J. Mol. Biol. 46, 329–335.