High-Pressure Effect on the Radical Reduction of Spin Probes in Human Erythrocytes

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The radical reduction of spin probes in human erythrocytes was studied using neutral radicals such as 2,2,6,6-tetramethyl-1-piperidinyloxyl (Tempo) and 2,2,6,6-tetramethyl-4-hydroxy-1-piperidinyloxyl (Tempol), and anion radical, 2,2,6,6-tetramethyl-4-phosphoryl-1-piperidinyloxyl (Tempo phosphate). On the basis of the differences in the reduction rate of spin probes in intact erythrocytes, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate-treated erythrocytes, and hemolysate, it was found that Tempo and Tempol permeate the lipid bilayer, while Tempo phosphate is transported by a band 3 protein. Under high presssure, the reaction of nitroxide radicals with reductants in cytosol was accelerated and the transport of the anion radical by the band 3 protein was suppressed.

Human erythrocyte membranes have been utilized in studies of various fields as a prototype of biological membranes. Ion transport across the plasma membrane is controlled by various integral proteins. Transport of Na^+ and K^+ is mediated by (Na^+–K^+)ATPase;[1] Ca^{2+} and Mg^{2+} ions are transported by (Ca^{2+}–Mg^{2+})ATPase;[2] and anions, such as Cl^- and HCO_3^-, are exchanged by a band 3 protein identified as being an anion channel.[3] These results concerning ion transport were obtained using mainly radio-labeled compounds. Since red blood cell contains various kinds of reductants inside the membrane, nitroxide radicals which are capable of permeating the membrane are readily reduced in the cytosol. This method has thus been used to estimate the membrane permeation of spin probes from the decrease of the ESR signal amplitude.[4]

The pressure as well as the temperature is a thermodynamic parameter that influences the rates and equilibria of chemical reactions. A study of a high-pressure effect on ion transport across the membrane has provided useful information concerning its transport mechanism. For instance, the transport of Na^+ and K^+ across the erythrocyte membrane is mediated by three components; the active transport by the sodium pump, the Cl^--dependent Na^+–K^+ cotransport system, and passive diffusion.[5] Na^+ and K^+ transport by the pump and cotransport system is suppressed by high pressure, whereas that by passive diffusion is significantly enhanced.[5] However, there have been few reports concerning small molecules, except for cations. We have thus attempted to examine the pressure effect on membrane permeation of neutral and anion molecules. By incubating neutral or anion nitroxide radicals with erythrocytes, the ESR signal amplitude is decreased. This reveals that nitroxide radicals permeate the membrane and then react with reductants contained in cytosol. Here, it is of interest to examine which process of membrane permeation and radical reduction is predominantly affected by high pressure. In the present work, we describe that under high pressure the reductive reaction of nitroxide radicals is accelerated and membrane permeation of the anion radical is suppressed.

Experimental

Materials. Neutral radicals, 2,2,6,6-tetramethyl-1-piperidinyloxyl (Tempo) and 2,2,6,6-tetramethyl-4-hydroxy-1-piperidinyloxyl (Tempol) were purchased from Aldrich Co. The anion radical, 2,2,6,6-tetramethyl-4-phosphoryl-1-piperidinyloxyl (Tempo phosphate), was synthesized according to the method of Weiner.[6] 4,4′-Diisothiocyanatostilbene-2,2′-disulfonate (DIDS) was from Wako Chemicals. All other chemicals were of reagent grade.

Membrane Permeation of Spin Probes. The heparinized blood from volunteer donors was centrifuged at 750×*G* for 10 min at 4 °C. The plasma and buffy coat were removed. The erythrocytes were washed three times in the phosphate buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 3.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 10 mM glucose, pH 7.0) and were packed by centrifugation at 750×*G* for 10 min at 4 °C. To modify the cells with DIDS, intact erythrocytes were incubated at 20% hematocrit in the phosphate buffer containing 0.1 mM DIDS for 30 min at 37 °C. DIDS-treated erythrocytes were washed three times in the phosphate buffer, and were packed as described above. Hemolysate was prepared by freezing and then thawing packed cells. In this hemolysate, the membrane structure of the erythrocyte is partially ruptured and the cell contents are released from the membrane. A radical solution containing 0.1 mM spin probes in the phosphate buffer was added to an equal volume of packed erythrocytes or hemolysate, and was then incubated at 37 °C. At a certain time interval, the aliquots were removed in order to measure the ESR spectra.

Membrane Permeation of Spin Probes under Pressure. Erythrocytes (or hemolysates) and radical solution were preincubated for 10 min at 37 °C and then mixed together. The samples were put into a syringe-type cell (0.3 ml) with a piston. The sample cell was placed in a pressure bomb made of stainless steel. Pressure was produced by means of a hand-type pump (Hikari Kikai) and monitored with a Heise pressure gauge. A mixture of ligroine and kerosene (v/v=1 : 1) was used as the pressure-transmitting fluid. The samples were compressed and decompressed at rates of 0.2 and 0.4 kbar min^{-1}, respectively.[7] ESR measurements of the samples were immediately carried out after decompression.

ESR Measurement. ESR measurements were carried out with a JES-RE1X spectrometer. The usual spectrometer settings were 100 kHz modulation amplitude, 0.05 mT; microwave power, 2 mW; scan range, 20 mT; scan speed, 8 min.

Results and Discussion

Figure 1 shows the ESR spectra of Tempol in an erythrocyte suspension. The ESR signal amplitude was decreased with the time course, but was restored by the addition of 0.5 mM $K_3[Fe(CN)]_6$, as was shown by Ross and McConnell[4] (data not shown). This indicates that the nitroxide radicals which penetrated through the membrane were readily reduced by such reductants as glutathione and ascorbic acid in cytosol. We therefore used A_t/A_o as a reduction parameter of spin probes, where A_o and A_t are the signal amplitudes of nitroxide radicals at 0 and t min after spin probe addition into the erythrocyte suspension (or hemolysate), respectively.

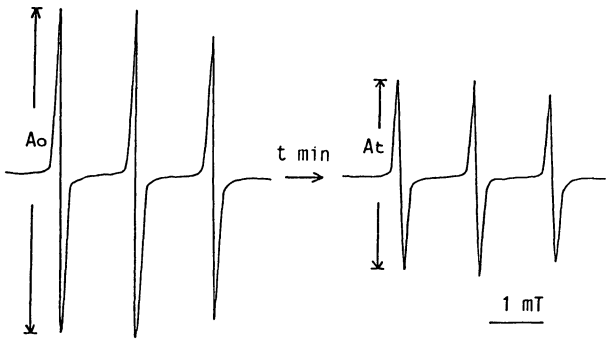

Fig. 1. ESR spectra of Tempol in erythrocyte suspension.

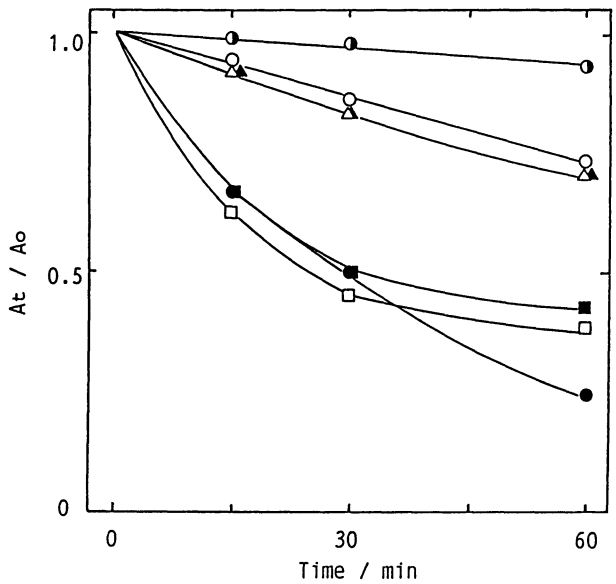

Fig. 2. Time course of the ESR signal amplitude of spin probes. Tempo phosphate (circles), Tempo (triangles), and Tempol (squares) were incubated with intact erythrocyte (open symbols), DIDS-treated erythrocyte (semiclosed symbols), and hemolysate (closed symbols).

Figure 2 shows the time course of the ESR signal amplitudes for three kinds of spin probes, i.e., Tempo, Tempol, and Tempo phosphate, in the erythrocyte suspension and the hemolysate. For neutral spin probes, such as Tempo and Tempol, the time course of A_t/A_0 in the erythrocyte suspension was almost the same as that in the hemolysate. On the other hand, the signal amplitude of Tempo phosphate in the hemolysate was remarkably decreased versus time compared to that in the erythrocyte suspension. In erythrocyte membrane, the transport of anions such as Cl^- and HCO_3^- is mediated by a band 3 protein well-known as an anion channel.[3] Thus, in order to obtain additional data concerning the membrane permeation of spin probes, intact erythrocytes were modified with DIDS, an anion transport inhibitor.[3] Under our conditions, it is expected that almost all of the bound DIDS is associated with the band 3 protein.[8] Membrane permeation of Tempo and Tempol was unaffected by DIDS (data not shown), whereas that of Tempo phosphate was significantly suppressed (Fig. 2). These results suggest that Tempo and Tempol can readily permeate the membrane, perhaps a phospholipid bilayer; the anion radical, Tempo phosphate, however, is transported by the band 3 protein. This suggestion is supported by the facts that such large-size anions as phosphoenolpyrvate and pyridoxal 5′-phosphate, which is comparable to Tempo phosphate in molecular size, are also transported by the band 3 protein.[9]

In the hemolysate, Tempo is difficult to reduce, compared to other spin probes. From the structural point of view, Tempo is the most hydrophobic; it is thus likely that Tempo enters the hydrophobic domains of the lipid bilayer and membrane proteins, and that it is inaccessible to reductants in cytosol. Furthermore, the stability of spin probes against reductants was estimated from the reaction of nitroxide radicals with ascorbate (0.1 mM) in aqueous solution. As with the hemolysate, the reduction of Tempo was most difficult (data not shown). Therefore, the slow decrease of the signal amplitude of Tempo in Fig. 2 may be ascribed to both the stability of the radical against reduction and its hydrophobicity.

Ion transport across the membrane is affected by high pressure.[5] When a human erythrocyte suspension is incubated under high pressure for 30 min at 37 °C, hemolysis starts to occur at 1.3 kbar.[10] In the present work, therefore, erythrocytes were subjected to pressures below the hemolysis pressure. Figure 3A shows the effect of high pressure on erythrocyte membrane permeation of spin probes. Upon the application of a pressure of 0.8 kbar to the erythrocyte suspension, the ESR signal amplitude of Tempo or Tempol was significantly decreased, whereas that of Tempo phosphate was increased. On the other hand, the signal amplitudes for all spin probes in the hemolysate were decreased at 0.8 kbar (Fig. 3B), indicating that the reductive reaction

of nitroxide radicals is facilitated under high pressure. At 0.8 kbar, the decrease in the signal amplitude of Tempo or Tempol in the erythrocyte suspension was almost the same as that of each spin probe in the hemolysate, as in the case at atmospheric pressure. This suggests that even in the erythrocyte under pressure the signal amplitude of Tempo or Tempol is controlled by the reaction rate of nitroxide radicals with the reductants contained in cytosol. The response of the signal amplitude of Tempol (or Tempo) to high pressure in an erythrocyte suspension is opposite to that of Tempo phosphate. The pressure dependence of the signal amplitude of Tempol and Tempo phosphate in the erythrocyte suspension was thus examined in the range from atmospheric pressure to 0.8 kbar (Fig. 4). With increasing pressure, the signal amplitude for Tempol linearly decreased, whereas that for Tempo phosphate gradually approached the level of no radical reduction. In general, chemical reactions characterized by a negative activation volume are greatly accelerated by high pressure. For instance, the Diels-Alder reaction exemplified in the cycloaddition reaction[11] and the cross-linking reaction of membrane proteins[10] are good examples of pressure effects. The glutathione, ascorbic acid, and mercapto groups in proteins may be major candidates for the reductants involved in cytosol. The reaction of Tempol with glutathione or ascorbate was thus examined under high pressure. In both cases, the rate of the reduction of nitroxide radicals was increased with increasing pressure (Fig. 5). However, the pressure effect on the reaction rate was more predominant in glutathione than in ascorbate. In each case, the reaction scheme is as follows:

$$2GSH + 2R\cdot \longrightarrow GS\text{-}SG + 2RH \quad (1)$$

and

$$AsOH + R\cdot \longrightarrow AsO + RH, \quad (2)$$

where GSH, AsOH, and R· are the glutathione, ascorbate, and nitroxide radicals, respectively. It is thus

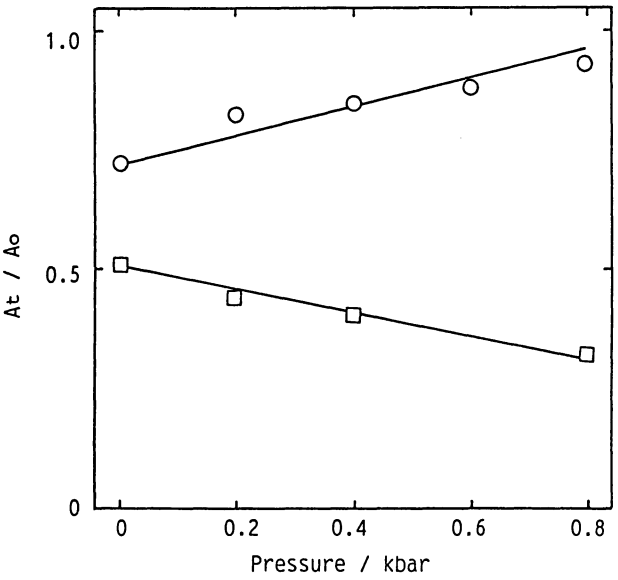

Fig. 4. ESR signal amplitudes of spin probes in an erythrocyte suspension as a function of the pressure. Tempo phosphate (○) and Tempol (□) were incubated for 60 and 30 min at 37 °C under pressures, respectively.

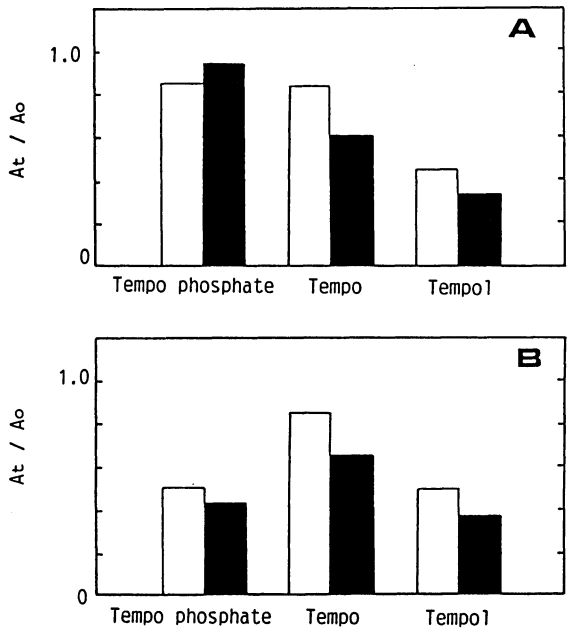

Fig. 3. Pressure effects on the ESR signal amplitude of spin probes in an intact erythrocyte suspension (A) and hemolysate (B). Samples were incubated for 30 min at 37 °C under atmospheric pressure (□) and 0.8 kbar (■).

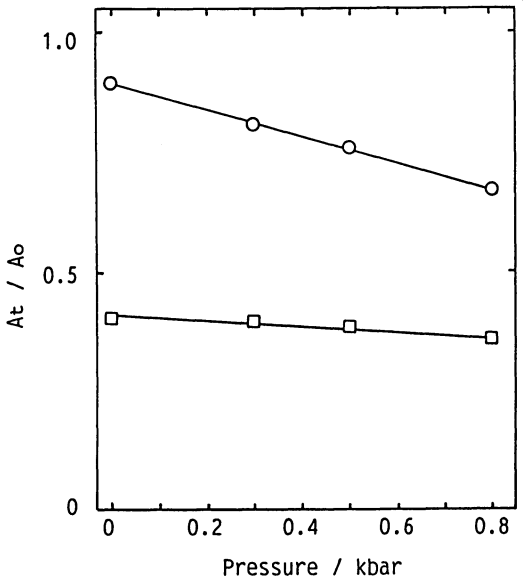

Fig. 5. ESR signal amplitude of Tempol (0.1 mM) incubated for 30 min at 37 °C under pressures in aqueous solutions containing reductants. ○, 0.1 mM glutathione; □, 0.1 mM ascorbate.

concluded that the reaction of nitroxide radicals with reductants contained in cytosol is substantially accelerated under high pressure. Additionally, the enhancement of the signal amplitude of Tempo phosphate with increasing pressure (Fig. 4) indicates that the membrane permeation of the anion radical is suppressed under high pressure.

Anion transport in human erythrocyte membrane is mediated by the band 3 protein.[3] A human band 3 protein (Mr 101791) comprises 911 amino acids,[12] and catalyzes a one-for-one exchange of anions across the plasma membrane. A conformational change of the band 3 protein from inward- to outward-facing forms, and vice versa, is an essential process for anion transport.[3] Such native conformational changes would be disturbed by the application of high pressure. It thus seems likely that anion transport by a band 3 protein is affected by high pressure. In fact, sulfate ($^{35}SO_4^{2-}$) equilibrium exchange is decreased at high pressure.[13] Using Tempo phosphate, we demonstrated that the transport of the anion radical by a band 3 protein is also significantly suppressed under high pressure.

In the present work, we have demonstrated that membrane permeation and radical reduction of nitroxide spin probes in human erythrocytes are affected by the application of high pressure. Additional kinetic information concerning them could be obtained by measuring the ESR spectra under high pressure.

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References

1) L. C. Cantley, *Curr. Top. Bioenerg.*, **11**, 201 (1981).
2) H. J. Schatzmann, *Annu. Rev. Physiol.*, **45**, 303 (1983).
3) H. Passow, *Rev. Physiol. Biochem. Pharmacol.*, **103**, 61 (1986).
4) A. H. Ross and H. M. McConnell, *Biochemistry*, **14**, 2793 (1975).
5) A. C. Hall, J. C. Ellory, and R. A. Klein, *J. Membr. Biol.*, **68**, 47 (1982).
6) H. Weiner, *Biochemistry*, **8**, 526 (1969).
7) T. Yamaguchi, H. Kawamura, E. Kimoto, and M. Tanaka, *J. Biochem.*, **106**, 1080 (1989).
8) A. Rothstein, Z. I. Cabantchik, and P. Knauf, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **35**, 3 (1976).
9) H. Nanri, N. Hamasaki, and S, Minakami, *J. Biol. Chem.*, **258**, 5985 (1983).
10) H. Kitajima, T. Yamaguchi, and E. Kimoto, *J. Biochem.*, **108**, 1057 (1990).
11) H. Takeshita, S. Sugiyama, and T. Hatsui, *Bull. Chem. Soc. Jpn.*, **58**, 2490 (1985).
12) S. E. Lux, K. M. John, R. R. Kopito, and H. F. Lodish, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9089 (1989).
13) V. A. Canfield and R. I. Macey, *Biochim. Biophys. Acta*, **778**, 379 (1984).