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Effects of intracellular pH on high pressure-induced hemolysis of anion transport inhibitor-treated erythrocytes

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Abstract

Effects of anion transport inhibitors such as 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate on hemolysis of human erythrocytes at 200 MPa were examined by changing intracellular pH (7.2–7.9). These inhibitors suppressed the hemolysis at neutral pH but enhanced it at alkaline pH. However, such an enhancement was suppressed by cross-linking of membrane proteins using diamide. From the near-UV CD spectra of band 3 and the relation between hemolysis and anion transport in intact or trypsin-treated erythrocytes, it was found that such hemolytic properties were characterized by the binding of inhibitors to band 3. In addition, spectrin detachment from the erythrocyte membrane by high pressure was considerably suppressed by DIDS treatment at neutral pH, but not by DIDS labeling at alkaline pH. These results suggest that the interaction of the cytoplasmic domain of band 3 with the cytoskeleton, which is induced by the binding of ligands to the exofacial domain of band 3, is dependent on the intracellular pH, i.e., the linking is tightened at neutral pH but relaxed at alkaline pH.

Keywords: Band 3; Erythrocyte; High pressure behaviour; Hemolysis; pH, intracellular

1. Introduction

Membrane stability of human erythrocytes is maintained by the interaction between transmembrane proteins and cytoskeletal ones. Band 3, which is the transmembrane protein and anion exchanger, is linked to β-spectrin via ankyrin [1–5]. Among other transmembrane proteins, glycophorin C plays an important role in the maintenance of the structural integrity. The cytoplasmic domain of glycophorin C interacts directly with protein 4.1 or indirectly via p55 with protein 4.1 [6–8]. Protein 4.1 is associated with spectrin and actin [9]. Thus, the phospholipid bilayer containing integral proteins interacts with the cytoskeleton via linking proteins such as ankyrin and protein 4.1. The abnormality in these protein-protein interactions results in the anomaly of cell shape and the membrane instability [10]. In band 3 from Southeast Asian ovalocytes (SAO), nine amino acids (residues 400–408) near N-terminal of the integral domain are deleted and the anion transport activity is lost [11]. In the SAO erythrocytes, the interaction of the cytoplasmic domain of band 3 with the cytoskeleton is altered as shown in membrane rigidity [11–14]. Hereditary spherocytosis is characterized by spectrin deficiency or reduction of spectrin content [15,16]. Leach phenotype erythrocytes are elliptocytic and a protein 4.1 content is reduced [7,17–19]. Therefore, these erythrocytes are more fragile.

Recently, it has been demonstrated that protein–protein interactions play an important role in signal transduction [20]. The binding of ligands to cell surface receptors induces the conformational changes of transmembrane proteins so that the interaction of the cytoplasmic domains of the receptors with adaptor proteins is mediated. For example, insulin binding to the insulin receptor induces tyrosine phosphorylation of the cytoplasmic domain of the receptor [21]. This autophosphorylation induces the phosphorylation of insulin receptor substrate-1 and protein–protein interactions via such phosphorylation cascade are transmitted to glycogen synthase [22]. From a signal transductional point of view, it is of interest to examine in more detail how the

Abbreviations: BSA, bovine serum albumin; $C_{12}E8$, octaethylene glycol mono-*n*-dodecyl ether; diamide, bis(*N*,*N*′-dimethylamide)diazinedicarboxylic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4; PLP, pyridoxal 5′-phosphate; PMSF, phenylmethanesulfonyl fluoride; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate.

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interaction of the cytoplasmic domain of a transmembrane protein with cytoskeletal proteins is mediated by the binding of the ligand to the extracellular portion of the transmembrane protein.

The analysis of the hemolysis provides the useful information about the interactions between erythrocyte membrane components. In particular, the degree of high pressure-induced hemolysis reflects sensitively the changes in membrane protein–protein interactions [23–25]. To characterize the binding of band 3 with cytoskeletal proteins via linking proteins, the effects of anion transport inhibitors on high pressure-induced hemolysis were examined. This paper focuses on the analysis of protein–protein interactions through hemolytic properties rather than a mechanism of the hemolysis. We demonstrate that the band 3-cytoskeletal protein interactions induced by anion transport inhibitors are significantly dependent on the intracellular pH.

2. Materials and methods

2.1. Chemicals

Compounds were obtained from the following sources: bis(*N*,*N*′-dimethylamide)diazinedicarboxylic acid (diamide), 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) and trypsin from bovine pancreas, Sigma; 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), Wako Chemicals; pyridoxal 5′-phosphate (PLP) and phenylmethanesulfonyl fluoride (PMSF), Nacalai Tesque; 4-maleimide-2,2,6,6-tetramethylpiperidinooxyl, Syva; octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$), Nikko Chemicals. All other chemicals were of reagent grade.

2.2. Chemical modification and enzymatic digestion of erythrocytes

Chemical modification and enzymatic digestion of the erythrocytes were performed at 20% hematocrit in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). To cross-link membrane proteins, the erythrocytes were incubated with 0.5 mM diamide for 30 min at 37°C under atmospheric pressure or 100 MPa [23]. To remove glycopeptides from the membrane surface, the erythrocytes were incubated with trypsin (0.1 mg/ml) for 1 h at 37°C [24]. These chemically and enzymatically modified cells were washed three times in PBS. For labeling with anion transport inhibitors, intact, diamide- or trypsin-treated erythrocytes were suspended at 20% hematocrit in PBS or 10 mM Tris, 150 mM NaCl (Tris buffer, pH 9.5), and incubated with DIDS (3–50 μM), SITS (50 and 250 μM) or PLP (5 mM) for 30 min at 37°C, unless otherwise noted. PLP-treated erythrocytes were incubated with 10 mM $NaBH_4$ in the presence of PLP for 15 min at 0°C. After incubation, red cells were washed three times in PBS. Intracellular pH was altered by incubating the erythrocytes at 10% hematocrit for 30 min at 37°C in Tris buffers of various pH values (pH 7.4–9.5).

2.3. Hemolysis

High pressure-induced hemolysis was examined, as previously described [25]. Briefly, the erythrocytes were suspended at 0.3% hematocrit in PBS and incubated for 30 min at 37°C under 200 MPa. After decompression, the suspension was centrifuged at 750 × *g* for 10 min at 35°C. For hypotonic lysis, the erythrocytes (10 μl at pellet) were added to 2 ml of 10 mM sodium phosphate (pH 7.4) containing 54 mM NaCl, incubated for 10 min at 37°C and centrifuged for 5 min at 1800 × *g*. The degree of hemolysis was estimated from the absorbance of hemoglobin released into the supernatant at 542 nm.

2.4. Estimation of anion transport and intracellular pH by ^{31}P-NMR measurement

Intact or trypsin-treated erythrocytes were suspended at 20% hematocrit in 5 mM sodium phosphate, 150 mM NaCl, pH 7.6 (buffer A), incubated for 60 min at 37°C and labeled with DIDS (3–50 μM) for 5 min at 0°C (Fig. 1A) or for 30 min at 37°C (Fig. 1B). The erythrocytes were washed three times in chilled buffer A (Fig. 1A) or twice with buffer A containing 0.5% BSA and then three times in chilled BSA-free buffer A (Fig. 1B). Inhibition of phosphate transport by DIDS was examined by measuring ^{31}P-NMR, as previously described [26]. Intracellular pH was estimated from the ^{31}P-NMR chemical shift of inorganic phosphate within the cell.

2.5. Conformational changes of band 3 induced by anion transport inhibitors

Isolation of band 3 from intact or anion transport inhibitor-treated erythrocytes was performed according to the method of Casey et al. [27]. The isolated band 3 was checked by SDS-PAGE and dialyzed overnight against 200 volumes of 5 mM sodium phosphate (pH 7.2) or 10 mM Tris (pH 9.5) buffer containing both 0.1% $C_{12}E_8$ and 0.1 mM PMSF prior to the near-UV CD measurement. Protein concentration was estimated from the absorbance at 280 nm using the specific absorptivity value of 1.55 lg^{-1} cm^{-1} [28]. The CD spectra of isolated band 3 were recorded at room temperature on a model J-600 spectropolarimeter (Jasco, Tokyo, Japan). The near-UV CD spectra were acquired with 2-nm/min scan speed, 1-nm band width, 10-mdeg sensitivity and 8-s time constant. These spectra were adjusted to zero CD at 320 nm. The molar ellipticities were obtained using a mean residue molecular mass of 111.7.

2.6. Spin labeling

Resealed ghosts which were prepared from intact or 50 μM DIDS-treated erythrocytes were incubated in PBS containing a maleimide spin label for about 7 h at 0°C [29] and washed three times in PBS. To change intracellular pH, spin-labeled ghosts were incubated for about 2 h at room temperature (23°C) in Tris buffer (pH 7.4 or 9.5) and washed once in chilled Tris buffer (pH 7.4). Intracellular pH was determined using ^{31}P-NMR, as mentioned above. The EPR spectra of spin-labeled ghosts were recorded at room temperature on a JEOL JES-RE-1X spectrometer. The usual spectrometer settings were 100-kHz modulation amplitude, 0.1 mT; microwave power, 20 mW; scan range, 20 mT; scan speed, 8 min.

2.7. Gel electrophoresis

The erythrocytes were preincubated for 30 min at 37°C in PBS or Tris buffer (pH 9.5) and treated with 50 μM DIDS for 30 min at 37°C in the same preincubation buffer. The red cells were washed three times in PBS. The erythrocyte suspension (5% hematocrit in PBS) was subjected to a pressure of 200 MPa for 30 min at 37°C. After decompression, the suspension was centrifuged for 10 min at 750 × g. The supernatant was further centrifuged at 4°C for 30 min at 81 000 × g. The supernatant was concentrated about 15-fold using Amicon ultrafiltration (PM-10 membrane). For a possibility of spectrin trapping within the membrane, high pressure-treated erythrocytes were exposed to 5 volumes of 5 mM sodium phosphate, pH 8.0 for 5 min at 0°C, hemolyzed and then mixed with the supernatant after decompression. Membrane-free hemolysates were similarly prepared by using ultracentrifugation and ultrafiltration, as described above. SDS-PAGE of concentrated samples was performed using 2.5% acrylamide (stacking gel) and 7% acrylamide (separation gel), according to the method of Laemmli [30]. Gel was stained with Coomassie blue.

3. Results

3.1. DIDS effects on hemolysis at 200 MPa and phosphate transport of intact or trypsin-treated erythrocytes

DIDS, anion transport inhibitor, binds specifically to the exofacial domain of band 3 [31]. So, from the DIDS effect on high pressure-induced hemolysis we attempted to evaluate the contribution of band 3 on the membrane stability of the erythrocyte. For DIDS labeling, the erythrocytes were treated with DIDS (3–50 μM) for 5 min at 0°C. The hemolysis at 200 MPa was considerably suppressed by DIDS (Fig. 1A). To examine whether the suppression is due to the DIDS binding to band 3, we tested a relationship between the hemolysis at 200 MPa and phosphate transport. In DIDS-treated erythrocytes, the percent hemolysis at 200 MPa was well correlated with the inhibition of phosphate transport (Fig. 1A, inset). For other anion transport inhibitors, similar suppressive effects were observed with SITS (% hemolysis at 50 μM is 22.8 ± 3.9 (n = 2)) but not with PLP (% hemolysis at 5 mM is 53.4 ± 1.8 (n = 2)). To avoid the reaction of DIDS with other membrane proteins such as glycophorins, the erythrocytes were exposed to trypsin. As demonstrated previously [24], the membrane structure of such erythrocytes became unstable to high pressure. However, upon DIDS treatment, the high pressure-induced hemolysis and phosphate transport were suppressed, as seen in intact cells (Fig. 1B).

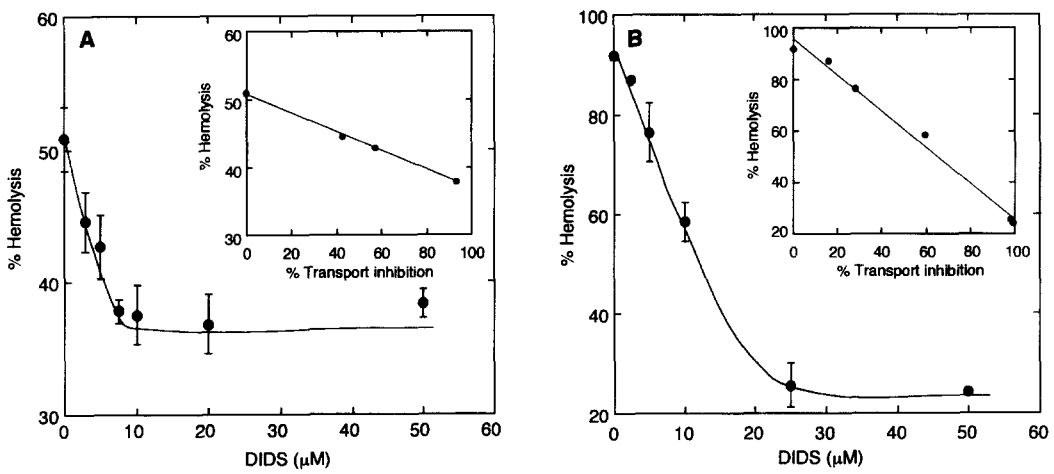

Fig. 1. Effects of DIDS on hemolysis at 200 MPa of intact and trypsin-treated erythrocytes. Intact erythrocytes (A) or trypsin-treated cells (B) were incubated with DIDS (3–50 μM) for 5 min at 0°C or for 30 min at 37°C, respectively. These erythrocytes in PBS were subjected to a pressure of 200 MPa for 30 min at 37°C. The inset shows the relationship between high pressure-induced hemolysis and phosphate transport inhibition. Values are the mean ± S.D. for at least two experiments.

3.2. Effects of intracellular pH on hemolysis at 200 MPa of DIDS-treated erythrocytes

Red cells were labeled with DIDS in a buffer of pH 7.4 (PBS). The DIDS-labeled cells were preincubated in buffers of various pH values (7.4–9.5) to alter the intracellular pH and subjected to a pressure of 200 MPa in PBS. The degree of hemolysis increased with rising buffer pH (Fig. 2) and responded reversibly to the changes in pH (data not shown). On the other hand, intact erythrocytes were also incubated in buffers of various pH values. However, the degree of hemolysis at 200 MPa was insensitive to the changes in pH (Fig. 2). Similar experiments were performed using anion transport inhibitors such as SITS and PLP. In this case, the enhancement of hemolysis by incubation at alkaline pH was observed in SITS (50 μM)-treated erythrocytes, but not in PLP (5 mM)-treated cells (data not shown).

From the ^{31}P chemical shift of inorganic phosphate within the cell, the intracellular pH was estimated. Intracellular pH of intact or DIDS-labeled erythrocytes which were incubated in buffers of various pH values (7.4–9.5) changed in a similar fashion, i.e., upon incubation for 30 min at 37°C in the buffer of pH 9.5, intracellular pH was about 7.6 in each case.

3.3. Effects of intracellular or extracellular pH during DIDS labeling on hemolysis at 200 MPa or in hypotonic buffer

To further characterize the effects of DIDS and intracellular pH on high-pressure-induced hemolysis, the DIDS labeling was carried out by changing intracellular or extracellular pH (Table 1). When intracellular pH was neutral (pH 7.2), the DIDS labeling was performed in the buffer of pH 7.4 or 9.5. In this case, the suppressive effect of DIDS appeared irrespective of extracellular pH. In contrast, when intracellular pH was alkaline (pH 7.9), the degree of hemolysis at 200 MPa increased upon DIDS labeling at pH 7.4 or 9.5. These results indicate that DIDS has a dual effect on high pressure-induced hemolysis depending on the intracellular pH, i.e., DIDS suppresses the hemolysis at neutral pH but enhances it at alkaline pH.

Table 1
Effects of intracellular and extracellular pH during DIDS labeling on hemolysis at 200 MPa

Intracellular pH	Extracellular pH	% Hemolysis	
		treatment	
		none	DIDS
7.2	7.4	44.2 ± 1.6	29.1 ± 3.9
7.2	9.5	38.4 ± 0.3	28.9 ± 2.5
7.9	7.4	41.0 ± 1.6	66.6 ± 4.0
7.9	9.5	45.7 ± 3.4	78.4 ± 5.3

Erythrocytes (10% hematocrit) were incubated for 30 min at 37°C in the buffer of pH 7.4 or 9.5 and then for 5 min at 37°C in the presence or absence of 50 μM DIDS. The erythrocytes were washed with chilled PBS and subjected to a pressure of 200 MPa for 30 min at 37°C. Values are the mean ± S.D. of at least two experiments.

The effect of intracellular pH during DIDS labeling on hypotonic lysis was examined. When the erythrocytes preincubated in Tris buffer of pH 7.4 were treated with 50 μM DIDS for 5 min at 37°C in the buffer of same pH, the degree of hypotonic lysis changed from 32.7 ± 2.4 ($n = 2$) to 66.9 ± 0.8 ($n = 2$). In the buffer of pH 9.5, similarly, the erythrocytes were preincubated and then labeled with 50 μM DIDS. DIDS-labeled cells were washed once in Tris buffer (pH 7.4). The degree of hypotonic lysis of DIDS-treated or -untreated erythrocytes was 24.2 ± 0.5 ($n = 2$) or 29.0 ± 3.2 ($n = 2$), respectively.

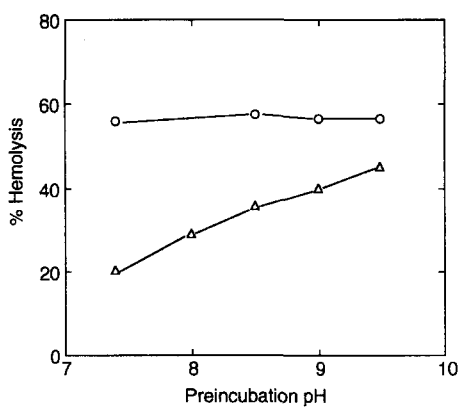

Fig. 2. Effects of preincubation pH on hemolysis at 200 MPa of intact and DIDS-treated erythrocytes. The erythrocytes were labeled with 50 μM DIDS in a buffer of pH 7.4 (PBS). Intact (○) and DIDS-labeled erythrocytes (△) were preincubated in Tris buffers (pH 7.4–9.5) for 30 min at 37°C and washed with PBS. These red cells were suspended in PBS and subjected to a pressure of 200 MPa for 30 min at 37°C.

Table 2
Effects of DIDS and labeling pH on hemolysis at 200 MPa of diamide-treated erythrocytes

Treatment			% Hemolysis
1st	2nd	3rd	
None	pH 7.4	DIDS	25.8 ± 1.8
None	pH 9.5	DIDS	77.6 ± 4.2
Diamide (0.1 MPa)	pH 7.4	DIDS	20.9 ± 0.4
Diamide (0.1 MPa)	pH 9.5	DIDS	79.7 ± 2.5
Diamide (100 MPa)	pH 7.4	DIDS	3.3 ± 0.8
Diamide (100 MPa)	pH 9.5	DIDS	7.2 ± 5.0

Erythrocytes (20% hematocrit in PBS) were treated with 0.5 mM diamide for 30 min at 37°C under 0.1 or 100 MPa. Diamide-treated cells were preincubated for 30 min at 37°C in the buffer of pH 7.4 or 9.5 and incubated with 50 μM DIDS for 30 min at 37°C. Thus, chemically modified cells were suspended in PBS and subjected to a pressure of 200 MPa for 30 min at 37°C. Values are the mean ± S.D. of at least two experiments.

3.4. Effects of DIDS and intracellular pH on hemolysis at 200 MPa of diamide-treated erythrocytes

The high pressure-induced hemolysis is enhanced by DIDS treatment when the intracellular pH is alkaline. So, we examined the effect of cross-linking of membrane proteins on such an enhancement (Table 2). The erythrocytes pretreated with diamide (0.5 mM) in PBS at atmospheric pressure were incubated in alkaline buffer (pH 9.5) and labeled with DIDS in the same pH buffer. In this case, the degree of hemolysis at 200 MPa increased upon DIDS labeling, as seen in intact erythrocytes. On the other hand, in red cells treated with diamide at 100 MPa, no such enhancement by DIDS was observed despite of intracellular alkaline pH.

3.5. Near-UV CD spectra of band 3

Band 3 was isolated from DIDS-, SITS- or PLP-treated erythrocyte membranes. SDS-PAGE of band 3 showed single band of 95-kDa (data not shown). To examine the conformational changes of band 3 induced by anion transport inhibitors, the near-UV CD spectra of band 3 solubilized in $C_{12}E_8$ were measured (Fig. 3). The optical activity in Phe/Tyr region below 280 nm decreased in DIDS- or SITS-labeled band 3 but did not in PLP-labeled band 3. In addition, DIDS-treated erythrocytes were exposed to alkaline pH (9.5) for 1 h at 37°C and then band 3 was isolated.

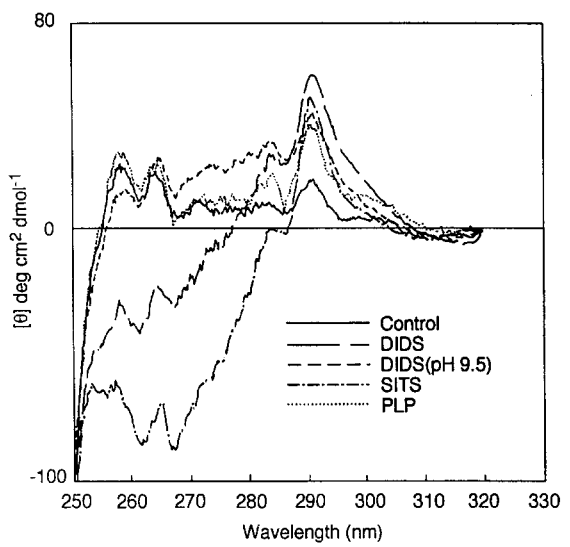

Fig. 3. Near-UV CD spectra of band 3. The erythrocytes (20% hematocrit in PBS) were treated with 50 μM DIDS, 250 μM SITS or 5 mM PLP for 30 min at 37°C. Aliquot of DIDS-treated erythrocytes was incubated for 1 h at 37°C in Tris buffer (pH 9.5). 5 mM PLP-treated cells were incubated with 10 mM $NaBH_4$ in the presence of PLP for 15 min at 0°C. Band 3 was isolated from these chemically modified cells as described in Section 2 and dialyzed against 5 mM sodium phosphate (pH 7.2) or 10 mM Tris buffer (pH 9.5) (for alkaline treatment of DIDS-treated erythrocytes) containing 0.1% $C_{12}E_8$ and 0.1 mM PMSF. The concentrations of band 3 were 0.5–1.2 mg/ml.

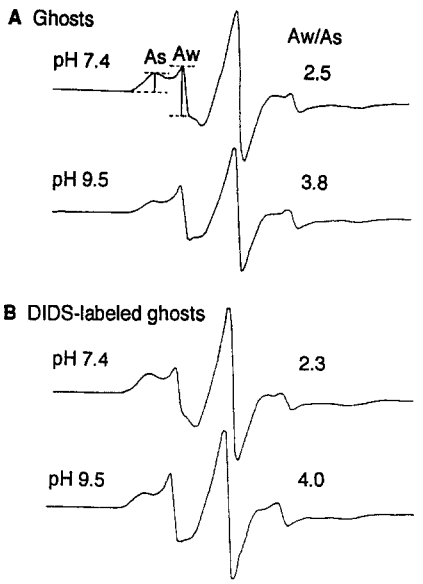

Fig. 4. Effects of intracellular pH on the EPR spectra of spin-labeled ghosts. Resealed ghosts prepared from intact or DIDS (50 μM)-treated erythrocytes were labeled with a maleimide spin label and washed with PBS. These spin-labeled ghosts were incubated for about 2 h at room temperature in Tris buffer (pH 7.4 or 9.5) and then washed once with chilled Tris buffer (pH 7.4).

The CD spectrum of such a band 3 approached to that of intact band 3. These results are similar to those reported by Batenjary et al. [28], except for the difference in the optical activity at 292 nm corresponding to tryptophan.

3.6. Conformational changes of membrane proteins induced by alkaline pH in spin-labeled ghosts

To examine the effect of intracellular pH on conformational changes of membrane proteins in DIDS-treated or -untreated ghosts, the membrane proteins were labeled with a SH-reactive maleimide spin label. When both ghosts were exposed to the buffer of pH 9.5 and then washed once in the buffer of pH 7.4, the values of intracellular pH were about 7.6. The EPR spectra of such ghosts showed the unfolding of membrane proteins, i.e., the values of conformational parameter (Aw/As) increased (Fig. 4).

3.7. DIDS effects on spectrin detachment from the erythrocyte membrane by high pressure

When intact erythrocytes are subjected to a pressure of 200 MPa, part of spectrin is detached from the membrane [25]. So, the effect of DIDS on spectrin detachment was examined by using SDS-PAGE (Fig. 5). Upon DIDS labeling at pH 7.4, the spectrin detachment by high pressure (200 MPa) was suppressed (Fig. 5, lanes 1 and 2). To check a possibility that any spectrin which is detached from the membrane remains trapped inside the cells, high pressure-treated erythrocytes were completely hemolyzed in the buffer of low ionic strength. SDS-PAGE of mem-

Fig. 5. Effects of DIDS and pH on spectrin detachment from the erythrocyte membrane by high pressure. The erythrocytes exposed to the buffer of pH 7.4 or 9.5 were incubated in the presence or absence of 50 μM DIDS in each buffer. Such cells were suspended in PBS and subjected to a pressure of 200 MPa for 30 min at 37°C. After decompression, the suspensions were centrifuged and the supernatants were concentrated by ultrafiltration. Aliquots of concentrated samples were applied to SDS-PAGE. Gel was stained with Coomassie blue.

brane-free hemolysates thus obtained also demonstrated that the spectrin detachment was suppressed by the DIDS treatment (data not shown). In contrast, no such suppressive effect by DIDS was observed in the membrane labeled by DIDS in the buffer of pH 9.5 (Fig. 5, lanes 3 and 4).

4. Discussion

When human erythrocytes are incubated with anion transport inhibitors such as DIDS and SITS, the major binding site of these inhibitors is band 3 [5,31,32]. To label specifically band 3, red cells were incubated with DIDS at a low temperature (0°C) for a short time (5 min) [32] or were digested with trypsin prior to DIDS labeling. Upon treatment with trypsin, the exofacial domain of glycophorins is digested [24,33] but band 3 remains stable [34]. In these erythrocytes, the suppression of high pressure-induced hemolysis is closely associated with the inhibition of phosphate transport (Fig. 1). Therefore, it seems likely that the membrane structure under high pressure is stabilized by the DIDS (or SITS) binding to band 3.

To characterize high pressure-induced hemolysis, we have also examined the hypotonic lysis. Upon removal of glycopeptides or sialic acid from the membrane surface by trypsin or neuraminidase, high pressure-induced hemolysis is enhanced but hypotonic lysis is not almost affected [24]. In addition, high pressure-induced hemolysis is greatly suppressed by cross-linking of transmembrane proteins with cytoskeletal proteins, whereas hypotonic lysis is not sensitive to such a chemical modification of red cells [23]. In the present work, we have shown that the effect of anion transport inhibitors on hemolysis is quite different in two methods, i.e., when high pressure-induced hemolysis is suppressed by DIDS, hypotonic lysis is enhanced, and vice versa. These data suggest that the mechanism of high pressure-induced hemolysis is quite different from that of hypotonic lysis. High pressure-induced hemolysis is more sensitive to the changes in protein–protein interactions.

High pressure-induced hemolysis at intracellular neutral pH is suppressed by DIDS and SITS but not by PLP, although anion transport is inhibited by these inhibitors. This indicates that the inhibition of anion transport is not associated with the suppression of high pressure-induced hemolysis. Similar conclusions are also obtained from the pH dependence of the hemolysis, as described later. Near-UV CD spectra of band 3 isolated from anion transport inhibitor-treated erythrocytes demonstrate that the tertiary structure of band 3 is altered by DIDS or SITS but not by PLP [28]. Here, it is of interest to examine how the interaction of band 3 with the cytoskeleton is mediated by these inhibitors. Membrane vesicles which are released from erythrocyte ghosts by DMPC liposomes contain spectrin in addition to band 3. However, the spectrin content decreases upon DIDS labeling of the erythrocyte membrane but does not change upon PLP labeling (Yamaguchi, T. et al., unpublished data). In high pressure-treated erythrocytes, part of spectrin is detached from the membrane [25]. The present work shows that such a detachment of spectrin is suppressed by DIDS. These data suggest that high pressure-induced hemolysis is suppressed by a tight interaction of the cytoplasmic domain of band 3 with the cytoskeleton induced by DIDS binding to the exofacial domain of band 3.

DIDS and 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS) have both NCS-groups [5]. So, it is useful to consider their reactivity and suppressive effect on hemolysis. At neutral pH, one NCS-group of H_2DIDS reacts readily with Lys-539 of band 3, whereas at alkaline pH another NCS-group reacts with Lys-851 [35,36]. However, such a reaction at alkaline pH does not take place for SITS and is difficult for DIDS [37]. As the intracellular pH becomes alkaline, on the other hand, the degree of hemolysis at 200 MPa of DIDS- or SITS-treated erythrocytes increases. Therefore, we exclude the contribution of Lys-851 to the hemolytic properties observed at alkaline pH.

Band 3 interacts with spectrin via ankyrin [1–5]. The binding of ankyrin to the cytoplasmic domain of band 3 is pH-dependent, i.e., at alkaline pH, the affinity of ankyrin to band 3 decreases [38,39]. In addition, the complex of spectrin with protein 4.1 dissociates at alkaline pH [40]. Thus, membrane protein–protein interactions within the red cell may be modulated at alkaline pH. Here, it is of interest to examine the pH effect on the interaction of

DIDS-labeled band 3 with cytoskeletal proteins. At neutral pH, the tertiary structure of band 3 is altered by DIDS, as shown in the near-UV CD spectra, so that the cytoplasmic domain of band 3 interacts tightly with the cytoskeleton. Upon exposure to alkaline pH of this DIDS-treated erythrocyte, the tertiary structure of DIDS-labeled band 3 tends to return to that of native band 3 [28] and such tight interaction is relaxed. Further, the pH dependence of pressure-induced hemolysis suggests that the interaction of band 3 with the cytoskeleton is reversibly mediated by pH.

To obtain an additional information on the conformation of membrane proteins, the SH groups of membrane proteins in ghosts were spin-labeled with a maleimide spin label. About 75% of spin labels is located to the spectrin-actin complex [41]. Upon exposure to alkaline pH of control ghosts or DIDS-labeled ones, the conformations of membrane proteins in both samples change similarly to unfolding state at intracellular alkaline pH. Similar results are obtained by heating (52°C) DIDS-treated erythrocytes, i.e., unfolding of membrane proteins, perhaps dissociation of spectrin tetramer to dimer [42], occurs and the suppressive effect of DIDS on hemolysis is removed (Yamaguchi, T. et al., unpublished data). Thus, the tight interaction of band 3 with the cytoskeleton induced by DIDS or SITS is relaxed under such conditions as membrane proteins unfold, i.e., alkaline pH and heating at 52°C.

When DIDS reacts with band 3 at intracellular alkaline pH, the degree of hemolysis at 200 MPa greatly increases. At alkaline pH, spectrin detachment from the membrane by high pressure is not suppressed by DIDS. Under such conditions as the affinity of ankyrin to band 3 decreases, the binding of DIDS to band 3 further weakens the interaction of band 3 with the cytoskeleton via ankyrin. Thus, the membrane structure becomes more fragile to high pressure. Here, it is of interest to see the effect of cross-linking of membrane proteins on such a fragility. In erythrocytes treated with diamide at atmospheric pressure, spectrin is mainly cross-linked [23]. The degree of hemolysis of such cells at 200 MPa increases upon DIDS labeling at alkaline pH, suggesting the inefficiency of the cross-linking of spectrin only. However, if the cytoplasmic domain of band 3 is cross-linked with cytoskeletal proteins prior to DIDS labeling, no such enhancement of hemolysis may be observed. When the erythrocytes are incubated with diamide at 100 MPa, large-molecular-weight polymers due to cross-linking of transmembrane proteins with the cytoskeleton are formed [23,24]. In this case, the membrane structure of such red cells remains stable against high pressure in spite of DIDS labeling at alkaline pH.

There are several reports that the binding of DIDS to the exofacial domain of band 3 induces the conformational changes of the cytoplasmic domain of band 3 [43–45]. For instance, Hsu and Morrison have shown that the interaction of band 3 with spectrin via ankyrin is strengthened by the binding of DIDS to band 3 [45]. We have demonstrated that DIDS has a dual effect on the interaction of band 3 with the cytoskeleton depending on the intracellular pH, i.e., the interaction is tightened at neutral pH but is weakened at alkaline pH. Thus, upon ligand binding to the exofacial domain of band 3, the interaction of the cytoplasmic domain of band 3 with the cytoskeleton becomes sensitive to pH. The present data suggest that the interactions of the cytoplasmic domain of receptor with adaptor proteins may be greatly modulated by some factors such as intracellular pH when ligands bind to the receptor.

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