A Structural Study of the Membrane Domain of Band 3 by Tryptic Digestion

**CONFORMATIONAL CHANGE OF BAND 3 IN SITU INDUCED BY ALKALI TREATMENT**

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It is well known that arginine, lysine, and glutamic acid are essential amino acids for band 3-mediated anion transport (Knauf and Rothstein, 1971; Cabantchik and Rothstein, 1974; Zaki et al., 1975; Zaki, 1981; Jennings, 1982; Wieth et al., 1982; Passow, 1986; Jennings and Anderson, 1987). Although the complete sequence of the human erythrocyte band 3 protein has been deduced from the cDNA sequence (Tanner et al., 1988; Lux et al., 1989), little is known about the nature and location of the functionally important amino acids. The site of inhibition of anion-exchange has been investigated by amino acid modification with pyridoxal phosphate on suitable proteolytic fragments of band 3 in combination with flux measurements (Hamasaki et al., 1983; Kawano and Hamasaki, 1986). The inhibitory site was identified as Lys-851 in the deduced sequence (Kawano et al., 1988), and this amino acid is the only one which is well characterized in anion transport.

In addition to the above amino acids, we have shown that an intracellular histidine residue of band 3 also participates in anion transport (Matsuyama et al., 1986). The intracellular histidine residue was modified by DEPC and extracellular binding of DNDS to band 3 prevented this DEPC modification (Izuhara et al., 1989), suggesting that this histidine residue is hidden from the intracellular surface of the membrane in its outward conformation and is thereby protected from DEPC modification by extracellular DNDS binding (Hamasaki et al., 1989).

In order to elucidate the topology of the functionally important amino acid residues within band 3, we have tried to assess the hydrodynamics of band 3 treated with bovine trypsin to digest the glycophorins. After washing, red blood cells were lysed osmotically with more than 20 volumes of low ionic strength solution, i.e. 5 mM sodium phosphate, pH 8 (3P8).

Experimental procedures

**Preparation of Leaky Human Erythrocyte Membranes (White Ghosts)—**Human blood which was stored for less than 2 weeks was used in these studies. The stored blood was obtained from the Fukuoka Red Cross Blood Center where it had been maintained in a citrate/phosphate/dextrose solution at 4 °C. Erythrocytes were treated with bovine trypsin to digest the glycophorins. After washing, red blood cells were lysed osmotically with more than 20 volumes of low ionic strength solution, i.e. 5 mM sodium phosphate, pH 8 (3P8).

Membranes (white ghosts) were centrifuged at 24,000 g for 30 min at 4 °C in a Hitachi RR 18A centrifuge and washed with the same buffer. The packed ghosts were stored at −40 °C until they were used.

**Analysis of Peptides Released from Membranes by Trypsin—**Ghosts (1 mg/ml) in 5P8 containing 0.14 M NaCl were digested with 15 μg/ml of trypsin at 4 °C for 1 h with stirring (referred to hereafter as...

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The abbreviations used are: DEPC, diethylpyrocarbonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; H-DIDS, dihydro-4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; 5P8, 5 mM sodium phosphate buffer, pH 8.
trypsin treatment with high salt concentration); the cytosolic 40,000-
dalton domain of band 3 was thereby cleaved. After addition of
antipain (10 μg/ml), the treated membranes were separated by cen-
trifugation (45,600 × g for 20 min at 4 °C) and washed extensively
with the same buffer. Peripheral membrane proteins in the washed
membranes were treated with 50 volumes of 0.1 M NaOH at
30 min and centrifuged at 45,600 × g for 20 min at 4 °C. The alkali-
stripped membranes were washed with 5P8 three times and resus-
pended in 5P8 at a protein concentration of 1.5 mg/ml. The mem-
brane suspension was redigested with 15 μg/ml of trypsin at 37 °C
for 30 min (i.e., trypsin treatment with low salt concentration).
Peptides released into the supernatant were collected by centrifug-
ation at 27,200 × g for 30 min at 4 °C. Under these conditions,
supernatants were clear and had no membranes.

The filtered supernatant was analyzed by high performance liquid
chromatography (HPLC) equipped with a reversed phase column
(Cosmosil C-18, 4.6 × 250 mm), using a linear gradient of 0–100%
acetonitrile containing 0.1% trifluoroacetic acid. This was the stan-
dard HPLC procedure for chromatographing peptides released into
the supernatant.

Analysis of Peptides Remaining in the Membranes after Tryptic
Digestion—White ghosts were treated with 5 volumes of 0.1 M NaOH
at 4 °C for 30 min to remove peripheral proteins. The alkali-stripped
membranes in 5P8 (1 mg/ml) were digested by 200 μg/ml of trypsin
at 37 °C for 1 h. After centrifugation at 27,200 × g for 30 min at 4 °C,
the membranes were resuspended in 5P8 containing 10 μg/ml of anti-
K5 antibodies and peptides were washed out from the mem-
branes with 5P8. The digested membranes were dissolved in an equal
volume of 8 M urea and 4% SDS containing 0.05 M dithiothreitol and
10% (v/v) formic acid, and warmed at 60 °C for 5 min. Peptides in
the dissolved membrane solution were separated on a Cosmosil C-8
column (4.6 × 300 mm) using a linear gradient of 40–100% acetonitrile
containing 0.1% trifluoroacetic acid.

Treatment of White Ghosts with Various Concentrations of NaOH—
White ghosts in cold water were mixed with 6 volumes of ice-cold
NaOH solution varying its concentration from 10 to 100 mM. The mixture of white ghosts and NaOH was immediately centrifuged at
45,600 × g for 4 °C. The pellet was washed three times with 40 volumes of 5P8 by centrifugation at 45,600 × g for 20 min at 4 °C.
The alkali-treated membranes were suspended in 5P8 at a protein
concentration of 1.5 mg/ml and digested with 15 μg/ml of trypsin
at 37 °C for 30 min as described above. Peptides released into the
supernatant were collected by centrifugation at 27,200 × g for 30 min
at 4 °C and analyzed by the standard HPLC procedure. Peripheral
membrane proteins were completely extracted from white ghosts
when the membranes were treated with 10 mM NaOH as shown in
Fig. 3D.

Peptide Synthesis and Antibody Production—The multiple antigen
peptides of KS-1 and KS-4 (which correspond to amino acid residues
from Lys-517 to Arg-627 and from Arg-646 to Arg-656, respectively,
in the deduced sequence of human erythroid band 3) were synthesized
using a peptide synthesizer (431 A, Applied Biosystems, Foster City,
CA) in accordance with the method of Tam (Tam, 1988; Tam and
Lu, 1988). The synthesized crude peptides were solubilized by 50%
acetic acid and separated by HPLC equipped with a reversed-phase
column (Chromosorb 300–7C4, 4.6 × 300, Chomcopak, Osaka, Japan)
using a linear gradient of 0–100% acetonitrile containing 0.1% triflu-
oroacetic acid. The molecular weight and amino acid composition of
the purified peptides examined were as anticipated (results not
shown).

Results—Except for the initial 200-μg dose, 100 μg/rabbit of each multiple
antigen were used with Freund’s adjuvant to immunize rabbits five
times every 2 weeks. The binding of each antiserum to erythrocyte
membranes was measured as follows. Ten μg of membranes/well were
attached to plates (Sumitomo multiplates MS-3596F/H, Tokyo, Ja-
pan). The indicated volume of serum in 100 μl of PBS containing
0.5% bovine serum albumin was added per well and allowed to stand
for 1 h at 37 °C. After washing with PBS three times, 100 μl of PBS
containing horseradish peroxidase-conjugated goat anti rabbit IgG
(E-Y Laboratories, San Mateo, CA) and 0.5% of bovine serum albu-
min were added, and the mixture was incubated at 37 °C for 1 h. The
content of bound IgG to membranes was assayed by a peroxidase
reaction using o-phenylenediamine as a substrate at 492 nm. There
was no cross-reaction between the site-specific antibodies.

Antipain—The multiple antigen peptides of KS-1 and KS-4 (which correspond to amino acid residues from Lys-517 to Arg-627 and from Arg-646 to Arg-656, respectively, in the deduced sequence of human erythroid band 3) were synthesized using a peptide synthesizer (431 A, Applied Biosystems, Foster City, CA) in accordance with the method of Tam (Tam, 1988; Tam and Lu, 1988). The synthesized crude peptides were solubilized by 50% acetic acid and separated by HPLC equipped with a reversed-phase column (Chromosorb 300–7C4, 4.6 × 300, Chomcopak, Osaka, Japan) using a linear gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid. The molecular weight and amino acid composition of the purified peptides examined were as anticipated (results not shown).

Analytical Procedures—SDS-PAGE for protein analysis was carried
out according to the method of Laemmli (1970). Protein was
determined by the method of Lowry et al. (1951) using bovine serum
albumin as the standard. Radioactivity was determined in sliced gels
with a liquid scintillation spectrometer. Peptides were sequenced on
a gas-phase sequencer (Applied Biosystems, model 470A), and the
phenylthiohydantoin derivatives were identified by an Applied Biosystems 120 A phenylthiohydantoin analyzer on-line system.

Materials—[3H]HID (purchased from New England Nuclear Research
Development Corp. (Toronto, Canada). DIDS was obtained from Cal-
biochem. Other reagents were of analytical grade.

RESULTS

Peptides Released from Erythrocyte Membranes by Tryptic
Digestion—To simplify the analytical procedure, the cytosolic
40,000-dalton domain of band 3 was nicked and removed by
treating white ghosts with trypsin at 4 °C as described under
“Experimental Procedures.” Peripheral membrane proteins were
also removed with NaOH at 4 °C. Thus, the transmem-
branos 55,000-dalton domain of band 3 is the major compo-
nent remaining (Fig. 1). The treated membranes were further
digested with the same concentration of trypsin (trypsin with
low salt concentration) at 37 °C, and the peptides released
were analyzed by the standard HPLC procedure. Fig. 2 shows a
typical elution profile of a HPLC analysis. The major
peaks were collected, rechromatographed with the same
HPLC system, and the purified peptides were sequenced by a
gas-phase sequencer. At least five peptides of band 3 origin,
designated as KS-1–KS-5, were released into the supernatant
from the alkali-stripped membranes by trypsin treatment,
and the amino acid sequences were the same as the amino
acid sequences deduced from cDNA (Table 1) (Tanner et al.,
1988; Lux et al., 1989). This indicates that the released
peptides are included in the hydrophilic connector loops. Among
these peptides, KS-1, KS-2, and KS-4 contained 1 mol of histidine residue/mol of peptide.

Analysis of Peptides Remaining in the Membranes after Tryptic Digestion—Peptides remaining in the membranes after the trypsin treatment were also analyzed. Washed trypsinized membranes were dissolved in an equal volume of 8 M urea and 4% SDS containing 0.05 M dithiothreitol and 10% (v/v) formic acid, and this mixture was applied to a Cosmosil C-8 column. Four major peaks, KM-6-KM-9, were obtained (Fig. 2B). Following purification and analysis of their primary structures, all of the amino acid sequences were also found to be in complete agreement with the deduced amino acid sequences, and KM-6 contained a histidine residue (Table I). The 8,500-dalton peptide which comprises the pyridoxal phosphate-binding site of Lys-851, also contains a histidine residue (Kawano et al., 1988). Thus, we were able to isolate five out of six histidine-containing peptides in the transmembrane 55,000-dalton domain of band 3.

Effect of NaOH Concentration on Tryptic Digestion—In the course of these experiments, we realized that the susceptibility of band 3 in situ to trypsin was modified by treatment of the membranes with NaOH. When erythrocyte membranes, which had not been trypsinized at 4 °C to remove the cytosolic 40,000-dalton domain, were treated with 100 mM NaOH and digested with trypsin at 37 °C in the presence of a low salt concentration (see “Experimental Procedures”), many more peptides including KS-1~KS-5 were released from the membranes (Fig. 3A), in contrast to Fig. 2A. On the other hand, when these membranes were treated with 10 instead of 100 mM NaOH, KS-1, KS-2, and KS-4 peptides were not released from the membranes by tryptic digestion, and the amounts of KS-3 and KS-5 released were smaller (Fig. 3B). The primary structure of a peptide appearing at about 25 min of elution time in the HPLC profile was YQSSPAPKPDSSFYK corresponding to the deduced sequence associated with Tyr-347 to Lys-360 of the cytosolic 40,000-dalton domain and was designated as peak 25 (Fig. 3A). In Fig. 3, A and B, peak 25 was excised and released to the supernatant equally from both membranes irrespective of alkali concentration. Judging from the SDS-PAGE results, all of the band 3 molecules in the membranes were digested with trypsin (data not shown). We selected peak 25, therefore, as an internal standard and quantitatively analyzed trypsin susceptibility of band 3 by using peak 25 and KS-4. The amount of KS-4 released into the supernatant increased as the alkali concentration was raised, and all of the band 3 molecules at the KS-4 region were digested with trypsin when the membranes were pretreated with 100 mM NaOH (Fig. 3C). With the KS-1 and KS-2 peptides, only about 30–40% of the band 3 molecules appeared to be digested with trypsin (Fig. 3C).

All peripheral membrane proteins appeared to be extracted when white ghosts were pretreated with 10 mM as well as with 100 mM NaOH-treatment (Fig. 3D), indicating that the altered susceptibility to trypsin is not due to steric hindrance.
caused by peripheral proteins but may be due to conformational changes of band 3.

**DISCUSSION**

Nine peptides derived from the transmembranous 55,000-dalton domain of band 3 were purified and sequenced in this study. All of the sequences agreed completely with deduced sequences from cDNA of human erythroid band 3. The cleavage sites involved the amino groups of Gly-361, Tyr-390, Val-604, Leu-632, Gly-647, Gly-699, Ser-731, Ile-761, Tyr-818, and the carboxyl groups of Lys-639, Arg-656, Lys-743, and Lys-826 (Table I). Peptides of KS-1 (Tyr-818-Lys-826), KS-2 (Ser-731-Lys-743), KS-3 (Leu-632-Lys-639), KS-4 (Gly-647-Arg-656), and KS-5 (Gly-361-Arg-656) were parts of hydrophilic connector loops. These hydrophilic connector loops were not always susceptible to trypsin and were rather resistant to the cross-linking of band 3 in situ is induced by alkali treatment. This view is also supported by DIDS cross-linking experiments.

When intact erythrocytes are digested with chymotrypsin, band 3 splits into the 60,000- and 38,000-dalton domains. The split domains can be cross-linked by incubating the cells with H$_2$DIDS at pH 9.5 (Jennings and Passow, 1979). Although both DIDS and H$_2$DIDS are specific inhibitors of anion transport mediated by band 3, the cleaved band 3 domains were cross-linked by incubating cells with H$_2$DIDS but not by DIDS. In our study, however, the cross-linking of band 3 with DIDS was also observed by treating chymotrypsinized membranes with ice-cold NaOH. The amount of band 3 cross-linked by DIDS (Fig. 6) as well as the increase in trypsin susceptibility of band 3 (Fig. 3C) were both increased when NaOH concentration was raised from 10 to 100 mM. Thus, the DIDS cross-linking with band 3 increased by alkali treatment (Fig. 6) was inversely proportional to the [H]$^3$H$_2$DIDS binding ability to band 3 (Fig. 5).

Reactivity of Antibody to Alkali-treated Membranes—The conformational change of band 3 was also confirmed in experiments with antibodies against band 3. Site-specific antibodies were developed with the multiple antigen peptides of KS-1 and KS-4 in accordance with the method of Tam et al. (Tam, 1988; Tam and Lu, 1989). The antibodies against KS-1 (IgG$_{KS-1}$) and KS-4 (IgG$_{KS-4}$) specifically reacted to band 3 after immunoblotting of erythrocyte membranes, and there was no cross-reaction between the two antibodies. The reactivity of the antibodies, IgG$_{KS-1}$ and IgG$_{KS-4}$, to the membranes increased as NaOH concentration was raised (Figs. 7, A and B), indicating that the conformation of band 3 in situ is changed by treating the cell membranes with NaOH.
Isolation of Hydrophilic Connector Loops of Band 3

FIG. 3. Effects of alkali treatment on the amount of KS-1, KS-2, and KS-4 released into the supernatant. White ghosts in cold water were mixed with 6 volumes of ice-cold NaOH solution varying in concentration from 10 mM to 100 mM as described under “Experimental Procedures.” The alkali-treated membranes were suspended in 5P8 at a protein concentration of 1.5 mg/ml and digested with 15 μg/ml of trypsin at 37°C for 30 min. Peptides released into the supernatant were collected by centrifugation and analyzed by HPLC as described in Fig. 2A. The amino acid sequences of all major peaks in panel A were analyzed and are discussed in the text. The peaks numbered 1–5 were peptides derived from the 55,000-dalton domain of band 3 and designated as KS-1 to KS-5, respectively. A peak numbered 25 was digested from the cytosolic 40,000-dalton domain and was used as an internal standard for quantitative analyses of KS-1, KS-2, and KS-4. A HPLC analysis of peptides released into the supernatant from membranes pretreated with 100 mM NaOH solution. Panel B, HPLC analysis of peptides released into the supernatant from membranes pretreated with 10 mM NaOH solution. Panel C, quantitative analyses of KS-1, KS-2, and KS-4 released by trypsin. Amounts of peptides released from membranes or DIDS-treated membranes were analyzed quantitatively using the peak 25 as an internal standard. Each value is a mean of two independent experiments. Amounts of KS-1 ( ), KS-2 (△), and KS-4 ( ) released from membranes; amounts of KS-1 (○), KS-2 (△), and KS-4 (○) released from DIDS-treated membranes. Panel D, SDS-PAGE (9% acrylamide) analysis of control membranes (lane 1), membranes treated with 10 mM NaOH (lane 2), and membranes treated with 100 mM NaOH (lane 3).

proteinase when band 3 was not treated with higher concentrations of NaOH (Fig. 3C). The other peptides, KM-6 (Gly-699~), KM-7 (Tyr-390~), KM-8 (Val-604~), and KM-9 (Ile-761~), constituted membrane-spanning helices. These topological sites corresponded closely with the hydrophathy prediction of Passow (1986) on murine band 3 but did not fit in a part of the model of human band 3 (Lux et al., 1989). The portion of KS-2 from Ser-731 to Lys-743 in the deduced sequence was predicted as a membrane-spanning region by Lux et al. (1989), but this peptide was released from the membrane by tryptic digestion, signifying that this peptide is part of a hydrophilic connector loop. It seems that their model
Isolation of Hydrophilic Connector Loops of Band 3

of band 3 should be reviewed with respect to the region around the KS-2 peptide. The sidedness of these hydrophilic connector loops remains to be resolved because we used unsealed membranes in the present investigation.

The transport of phosphate and phosphoenolpyruvate across the erythrocyte membrane is mediated by band 3 (Hamasaki et al., 1983; Hamasaki and Kawano, 1987), and transport rates were inhibited when intracellular pH was lowered from pH 6.8 to 6.0, as if inhibition depended on protonation of groups with a pK of approximately 6.6 inside the cell membrane (Matsuyama et al., 1986). Diethyl pyrocar-bonate (DEPC), a histidine-oriented reagent, inhibited phosphate transport across the cell membrane only when membranes were modified with the reagent from the cytosolic surface of membranes (Izuhara et al., 1989). In addition, extracellular DNDS protected the intracellular amino acid from DEPC modification and, reciprocally, DEPC modification inhibited extracellular H$_2$DIDS binding to band 3 (Izuhara et al., 1989). Thus, it appears that the essential histidine residue(s) for anion transport is located on the cytosolic surface of band 3 and that extracellular binding of DNDS to band 3 induces a conformational change in the intracellular portion of band 3 such that the histidine residue(s) is hidden from the cytosolic surface of the cell membrane (Hamasaki et al., 1988).
FIG. 7. Reactivities of IgGKS-1 and IgGKS-4 to white ghosts and alkali-treated membranes. The multiple antigen peptides KS-1 and KS-4 which correspond to amino acid residues from Lys-819 to Arg-827 and from Arg-646 to Arg-656, respectively, in the deduced sequence of human erythroid band 3, were synthesized and antibodies against KS-1 and KS-4 were produced. The binding of each antiserum to erythrocytes membranes was measured at 492 nm as described under "Experimental Procedures." Each value is a mean of two independent experiments. Reactivity was tested in white ghosts (□), membranes treated with 10 mM NaOH (●), and membranes treated with 100 mM NaOH (○). Panel A, reactivity of IgGKS-1 to white ghosts and alkali-treated membranes. Panel B, reactivity of IgGKS-4 to white ghosts and alkali-treated membranes.

In order to identify the essential histidine residue within the band 3 molecule, we attempted to isolate peptides containing histidine residues. According to the cDNA sequence of human erythroid band 3 (Tanner et al., 1988; Lux et al., 1989), 6 histidine residues are expected in the 55,000-dalton domain of band 3. In the present paper, we were able to isolate nine peptides from the transmembrane domain of band 3. Among them, four peptides, KS-1, KS-2, KS-4, and KM-6, contained a histidine residue (Table 1). Another histidine residue was isolated with the acetylated 8,500-dalton peptide which contains Lys-851, the pyridoxal phosphate-binding site (Kawano et al., 1988; Okubo et al., 1991). As a consequence, we have been able to isolate histidine-containing peptides associated with 5 of the 6 histidine residues in the 55,000-dalton domain of band 3.

As shown in Fig. 3C, the amount of the KS-1, KS-2, and KS-4 peptide released by trypsin treatment increased as NaOH concentration was raised during alkaline treatment of the erythrocyte membranes. The change in trypsin susceptibility was not due to steric hindrance by peripheral membrane proteins because all of these proteins had been extracted when the membranes were treated with 10 mM NaOH; at this concentration none of the band 3 was digested by trypsin (Fig. 3C). Thus, the change in trypsin susceptibility appears to be due to the conformational change in band 3 induced by alkali treatment. Susceptibility of band 3 to carboxypeptidase Y was altered when cell membranes are treated with alkali (Lieberman et al., 1987), thereby suggesting also that the conformation of band 3 in situ may be changed by alkali treatment of cell membranes.

The DIDS-bound form of band 3 was resistant to conformational change induced by alkali treatment. The release of peptides by trypsin was strongly inhibited by the DIDS pretreatment (Figs. 3C and 4), indicating that DIDS binding to band 3 stabilizes the band 3 conformation which is resistant to alkali treatment. The DIDS-complexed form of band 3 is resistant to thermal denaturation (Appell and Low, 1982) as well as to papain digestion (Jennings et al., 1984), indicating that DIDS-stabilized band 3 conformation also resists these other influences.

The present paper indicates that the hydrophilic connector loops of band 3 containing histidine residues tend to be resistant to alkali-trypsin treatment when DIDS bound to band 3 and converted the configuration to the outward facing form. One of the mobile histidine residues included in the peptides of KS-1 (His-819), KS-2 (His-734), and KS-4 (His-651) could be the essential histidine residue for anion transport because this residue was hidden from the cytosolic surface of the cell membrane in the outward facing form and was thereby protected from the DEPC modification (Izuhara et al., 1989; Hamasaki et al., 1989). However, there was no direct evidence in this paper to indicate that the conformational change observed is related to the anion transport process.

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