Molecular Basis and Functional Consequences of the Dominant Effects of the Mutant Band 3 on the Structure of Normal Band 3 in Southeast Asian Ovalocytosis†

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ABSTRACT: Southeast Asian ovalocytosis (SAO) human red cell membranes contain similar proportions of normal band 3 and a mutant band 3 with a nine amino acid deletion (band 3 SAO). We employed specific chemical modification and proteolytic cleavage to probe the structures of band 3 in normal and SAO membranes. When the membranes were modified specifically at lysine residues with *N*-hydroxysulfosuccinimide-SS-biotin, band 3 Lys-851 was not modified in normal membranes but quantitatively modified in SAO membranes. Normal and SAO membranes showed different patterns of band 3 proteolytic cleavage. Notably, many sites cleaved in normal membranes were not cleaved in SAO membranes, despite the presence of normal band 3 in these membranes. The mutant band 3 changes the structure of essentially all the normal band 3 present in the SAO membranes, and these changes extend throughout the normal band 3 molecules. The results also imply that band 3 in SAO membranes is present as hetero-tetramers or higher hetero-oligomers. The dominant structural effects of band 3 SAO on the other band 3 allele have important consequences on the functional and hematological properties of human red cells heterozygous for band 3 SAO. Analysis of the altered profile of biotinylation and protease cleavage sites suggests the location of exposed surfaces in the band 3 membrane domain and identifies likely interacting regions within the molecule. Our approach provides a sensitive method for studying structural changes in polytopic membrane proteins.

The human red cell anion exchanger (band 3, AE1) is a multispanning polytopic membrane protein that transports anions in the red blood cells and the α-intercalated cells of the distal nephron of the kidney. The protein comprises two functional domains with distinct functions. The N-terminal cytoplasmic domain (amino acids 1−359) associates with peripheral membrane proteins and forms a membrane anchorage site for the red cell cytoskeleton (reviewed in refs *1, 2*). The C-terminal portion of the protein (amino acids 360−911) carries out anion exchange and contains 12 or 14 transmembrane (TM)[1] segments (see refs *3*−*7* for reviews). The major differences between models that contain 14 TM spans (for example, ref *8*) and models with 12 TM spans (*9*) stem from ambiguity in the topology of TM9−12 of the 14 span model.

Southeast Asian ovalocytosis (SAO; reviewed in ref *7*) results from the heterozygous presence of a mutant band 3 (band 3 SAO). SAO red cells are unusually rigid (*10*), and the condition is associated with resistance to cerebral malaria (*11*). Band 3 SAO contains a deletion of nine amino acids (residues 400−408) at the cytoplasmic boundary of the first transmembrane span (TM1) of normal band 3. Homozygosity for the abnormal gene appears to be lethal. Band 3 SAO does not transport anions (*12*). SAO red cell membranes contain 55% normal band 3 and 45% band 3 SAO (*13, 14*).

Previous studies have shown that, unlike TM1 of normal band 3, TM1 of band 3 SAO does not insert into microsomes in an alkali-stable fashion, but TM1−3 and larger fragments of band 3 SAO are incorporated stably into microsomal membranes (*15*). The structural effects of the SAO deletion extend at least over TM1−5 of the protein. Lys-430 in the loop between TM1 and TM2 (loop 1−2) of normal band 3 reacts with extracellular eosin maleimide, but the equivalent residue in band 3 SAO is not reactive (*16*). While normal band 3 covalently binds extracellular 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS) at Lys-539 and

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[1] Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; dRTA, distal renal tubular acidosis; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NHS-SS-biotin, *N*-hydroxysulfosuccinimide-SS-biotin; SAO, Southeast Asian ovalocytosis; TM, transmembrane spanning portion.

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cross-links it to Lys-851 (*17*), band 3 SAO does not bind extracellular H_2DIDS, either reversibly or irreversibly (*12*). However, the extracellular loop 5−6 of band 3 SAO is cleaved by chymotrypsin treatment of intact SAO red cells, like the normal protein. In addition, band 3 SAO is glycosylated at the normal site of N-glycosylation (Asn-642 in loop 7−8), but with an abnormal *N*-glycan chain (*15, 18*). The latter observations suggest that the gross topology of band 3 SAO in the regions of extracellular loops 5−6 and 7−8 remains the same as the normal protein. Band 3 forms dimers and tetramers, and one site of association between band 3 monomers is through the C-terminal membrane domain (*19*−*21*). Normal band 3 forms heterodimers with band 3 SAO in SAO red cell membranes (*22*).

The membrane domain of band 3 is relatively resistant to denaturation in situ in red cell membranes. After treatment with 10 mM NaOH (pH 12), the protein has limited susceptibility to trypsin and retains the ability to bind covalently the anion transport inhibitor H_2DIDS (*23*). Washing of membranes at pH 12 has been widely used to dissociate the cytoskeleton during the preparation of band 3. However, 100 mM NaOH treatment partially denatures the membrane domain of band 3 with loss of H_2DIDS binding and enhanced cleavage by proteases (*23, 24*). Detailed studies have been carried out on the sites of protease cleavage of the membrane domain of band 3, and the fragments liberated and retained in the membrane have been characterized (*24*).

In this paper, we have used chemical modification and proteolytic cleavage as probes to compare the structure of the membrane domain of band 3 in normal and SAO red cell membranes. Our results suggest that the band 3 SAO in SAO red cell membranes significantly modifies the structure of the normal band 3 in these membranes. Examination of the alterations in the profiles of proteolytic cleavage and chemical modification suggests likely interacting regions within the band 3 membrane domain monomer and oligomer. The dominant structural effects of band 3 SAO on the other band 3 allele explain the dominant effects of this protein on the functional and hematological properties of human red cells heterozygous for band 3 SAO.

EXPERIMENTAL PROCEDURES

Preparation of Human Red Cell Membranes and Chemical Modification of Band 3 Protein in Situ with N-Hydroxysulfosuccinimide-SS-biotin (NHS-SS-biotin). Red cell membranes (white ghosts) were prepared as described previously (*23, 24*). Briefly, red cells were pretreated with bovine trypsin to digest the glycophorins. White ghosts were prepared by osmotic lysis, and the cytosolic 40 kDa domain of band 3 protein was removed by TPCK-trypsin digestion (final concentration 15 μg/mL) for 30 min on ice. Peripheral membrane proteins were removed with 10 mM NaOH. Alkali treatment with 10 mM NaOH (pH 12) or 100 mM NaOH (pH 13) was done with 5 volumes of NaOH solution on ice for 30 min with stirring. Chemical modification with NHS-SS-biotin (final concentration 0.1−1.0 mM) of the NaOH-pretreated membranes was performed in 50 mM sodium bicarbonate buffer, pH 8.5, at 4 °C for 30 min. Preliminary experiments using bovine serum albumin confirmed that lysine residues were modified but cysteine residues were not modified under these conditions.

In Situ Proteolytic Digestion of Band 3 Protein Modified with NHS-SS-biotin. To assist proteolysis, we denatured band 3 in situ with 100 mM NaOH. The alkali-treated membranes were washed and resuspended in 5 mM phosphate buffer (pH 8.0) at a protein concentration of 1.0 mg/mL. The membrane suspension was digested with 15 μg/mL chymotrypsin at 4 °C for 30 min, followed by 15 μg/mL trypsin at 4 °C for 30 min. The chymotrypsin/trypsin-treated membranes were solubilized with an equal volume of 4 M urea/2% SDS.

HPLC Analysis of Modified and Fragmented Peptides. Peptides in the solubilized membranes were separated by HPLC using a reversed-phase column (Cosmosil 5C18-AR-300 or Cosmosil 5C18-MS, 4.6 × 250 mm, Chemco, Osaka, Japan), with a water/acetonitrile/propanol eluant system containing 0.1% trifluoroacetic acid. Water containing 0.1% trifluoroacetic acid was the eluant for the first 70 min and at 275 min. An eluant gradient was used between 70 and 275 min as follows: 70−75 min, water 100−90%, propanol 0−10%; 75−165 min, water 90−0%, acetonitrile 0−90%, propanol 10%; 165−245 min, acetonitrile 90−10%, propanol 10−90%; 245−255 min, acetonitrile 10%, propanol 90%; 255−265 min, water 0−90%, acetonitrile 10−0%, propanol 90−10%; 265−275 min, water 90−100%, propanol 10−0%. The flow rate of the elution buffer was 0.5 mL/min.

Fractions were collected every 2 min, and all fractions were analyzed by a gas-phase sequencer (Applied Biosystems, model 492, CA). The phenylthiohydantoins were identified with an Applied Biosystems 140C phenylthiohydantoin analyzer on-line system. All fractions were also analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a Voyager RP with BioSpectrometry workstation (PerSeptive Biosystems, Framingham, MA). All the HPLC fractions were collected, and every fraction was subjected to amino acid sequence analysis using both methods described above.

Analytical Procedures. SDS−PAGE was carried out as described (*25*). Protein was determined by the method of Lowry et al. (*26*) using bovine serum albumin as the standard.

RESULTS

Modification of the Available Lysine Residues in the Band 3 Membrane Domain by NHS-SS-biotin. The optimal concentration of NHS-SS-biotin required for modifying the available lysine residues in the band 3 membrane domain was determined. Figure 1 shows the modification of Lys-892 of band 3 in membranes pretreated with 10 mM NaOH. Maximal modification of this lysine residue was reached at 0.25 mM NHS-SS-biotin, with 14% of the residues modified. This concentration (0.25 mM) of NHS-SS-biotin also gave maximal modification of the other available lysine residues in band 3, but at maximal modification, each lysine residue was modified to a different extent. NHS-SS-biotin was used at 0.25 mM in the following experiments. Normal red cell ghosts treated with 10 mM NaOH or 100 mM NaOH were incubated with NHS-SS-biotin and digested with chymotrypsin and trypsin, and after HPLC separation, the NHS-SS-biotin bound to each peptide was semiquantitatively estimated by amino acid sequencing. The biotinylation modification in the peptides was confirmed by MALDI-TOF mass spectrometry. The biotinylation of Lys-814 and Lys-

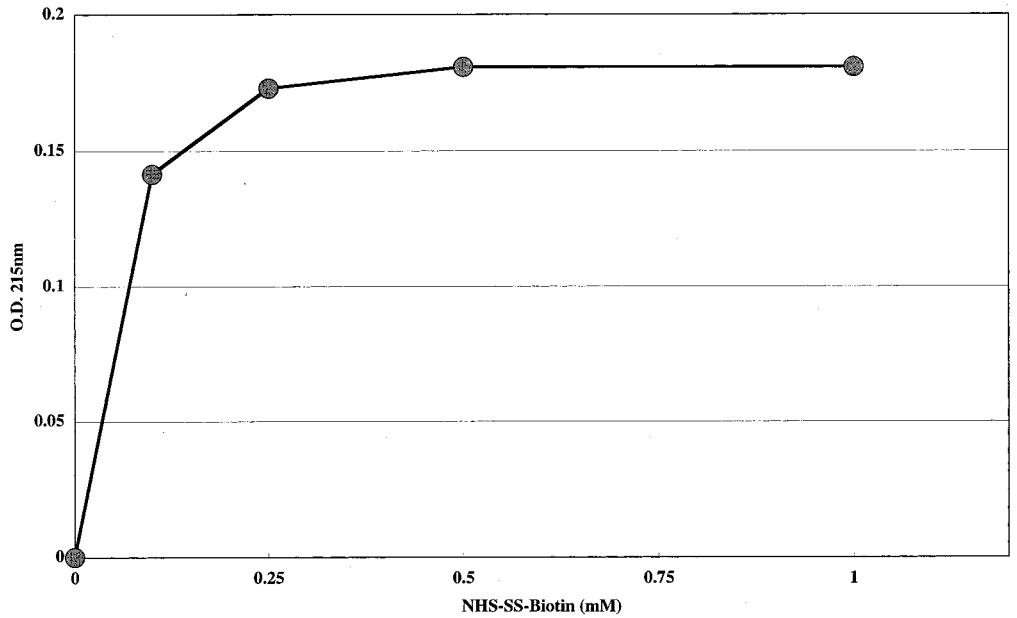

FIGURE 1: Modification of Lys-892 in band 3 by NHS-SS-biotin treatment of normal red cell membranes. Chemical modification with NHS-SS-biotin (final concentration 0.1−1.0 mM) of the band 3 protein in 10 mM NaOH-pretreated normal membranes was performed at 4 °C for 30 min, using 50 mM sodium bicarbonate buffer (pH 8.5). The modified membranes were treated with 100 mM NaOH and digested with chymotrypsin and trypsin, and the band 3 peptide fragment containing Lys-892 was separated by HPLC and analyzed by a gas-phase sequencer and by MALDI-TOF mass spectrometry as described under Experimental Procedures. The extent of modification of Lys-892 was estimated from the peak height of the band 3 peptide containing Lys-892 obtained on HPLC. An exactly similar titration curve was obtained for the modification of the peptide containing Lys-814/Lys-817.

817 could not be distinguished since the two lysine residues were isolated in the same peptide fragment. However, MALDI-TOF mass spectrometry showed this peptide maximally incorporated only 1 mol of NHS-SS-biotin per mole of peptide. Since it is not known whether only one of the lysine residues was modified or whether both lysine residues were partially biotinylated, biotinylation at one or both of these residues is indicated below by "Lys-814/Lys-817".

Figure 2(a,b) shows the results obtained after biotinylation of normal and SAO membranes pretreated with 10 mM NaOH. Only two lysine residues were modified in the normal band 3 sample: Lys-814/Lys-817 was 100% modified, and Lys-892 was 14% modified (Figure 2a). The band 3 in the SAO membranes showed a similar degree of modification of Lys-814/Lys-817 and Lys-892 (100% and 20%, respectively) but three additional lysine residues were reactive with the reagent: Lys-851 was 100% modified, while Lys-691 and Lys-757 were 40% and 30% modified, respectively (Figure 2b).

The biotinylation of normal and SAO red cell membranes pretreated with 100 mM NaOH is shown in Figure 2(c,d). In the normal sample, Lys-892 was modified to a similar extent (20%) as in the 10 mM NaOH sample, while the modification of Lys-814/Lys-817 was markedly reduced (to 30%) at the higher NaOH concentration (Figure 2c). The increased NaOH concentration also resulted in the exposure of additional lysine residues in normal band 3: Lys-430, Lys-631, Lys-691, and Lys-757 (modified by 50%, 40%, 35%, and 25%, respectively). The band 3 in the SAO membranes treated with 100 mM NaOH showed a biotinylation pattern similar to the normal protein except that both Lys-851 and Lys-814/Lys-817 were completely modified (Figure 2d).

Proteolysis of Normal and SAO Membranes Modified with NHS-SS-biotin. Red cell membranes pretreated with trypsin to remove the N-terminal cytoplasmic domain of band 3 were treated with 10 mM NaOH or 100 mM NaOH and then chemically modified with NHS-SS-biotin. These membrane preparations were further washed with 100 mM NaOH and digested with chymotrypsin and trypsin, and the peptide fragments produced were separated by HPLC. Parallel experiments were carried out using normal and SAO red cell membranes (each containing 2500 pmol of band 3). Figure 3a shows the HPLC profile obtained for the normal membrane sample modified with NHS-SS-biotin under native conditions (10 mM NaOH pretreated membranes), and Figure 3b shows the profile obtained from the SAO membranes under the same conditions. Figure 3c and Figure 3d show the HPLC profiles obtained from normal and SAO red cell membranes, respectively, modified by the biotinylation reagent after denaturation by 100 mM NaOH (100 mM NaOH pretreated membranes). Although the normal and SAO membrane samples show similarities in the HPLC profiles, detailed analysis of the separated peptide fragments by MALDI-TOF mass spectrometry and amino acid sequencing demonstrated differences in the sites of cleavage in normal band 3 and the band 3 SAO. Details of the peptide sequences recovered are provided in Supporting Information Tables 1−6 (see Supporting Information). The locations of the protease cleavage sites and the amount of cleavage at each site for the normal and SAO membranes treated with 10 mM NaOH or 100 mM NaOH before modification by NHS-SS-biotin are shown in Table 1 and schematically in Figure 4. The results obtained for the normal 100 mM NaOH pretreated membranes were consistent with the fragmentation patterns previously reported (*17*, *24*) and were reproducible.

Proteolysis of Normal and SAO Red Cell Membranes Treated with 10 mM NaOH. There were relatively few sites of protease cleavage in either normal or SAO membrane

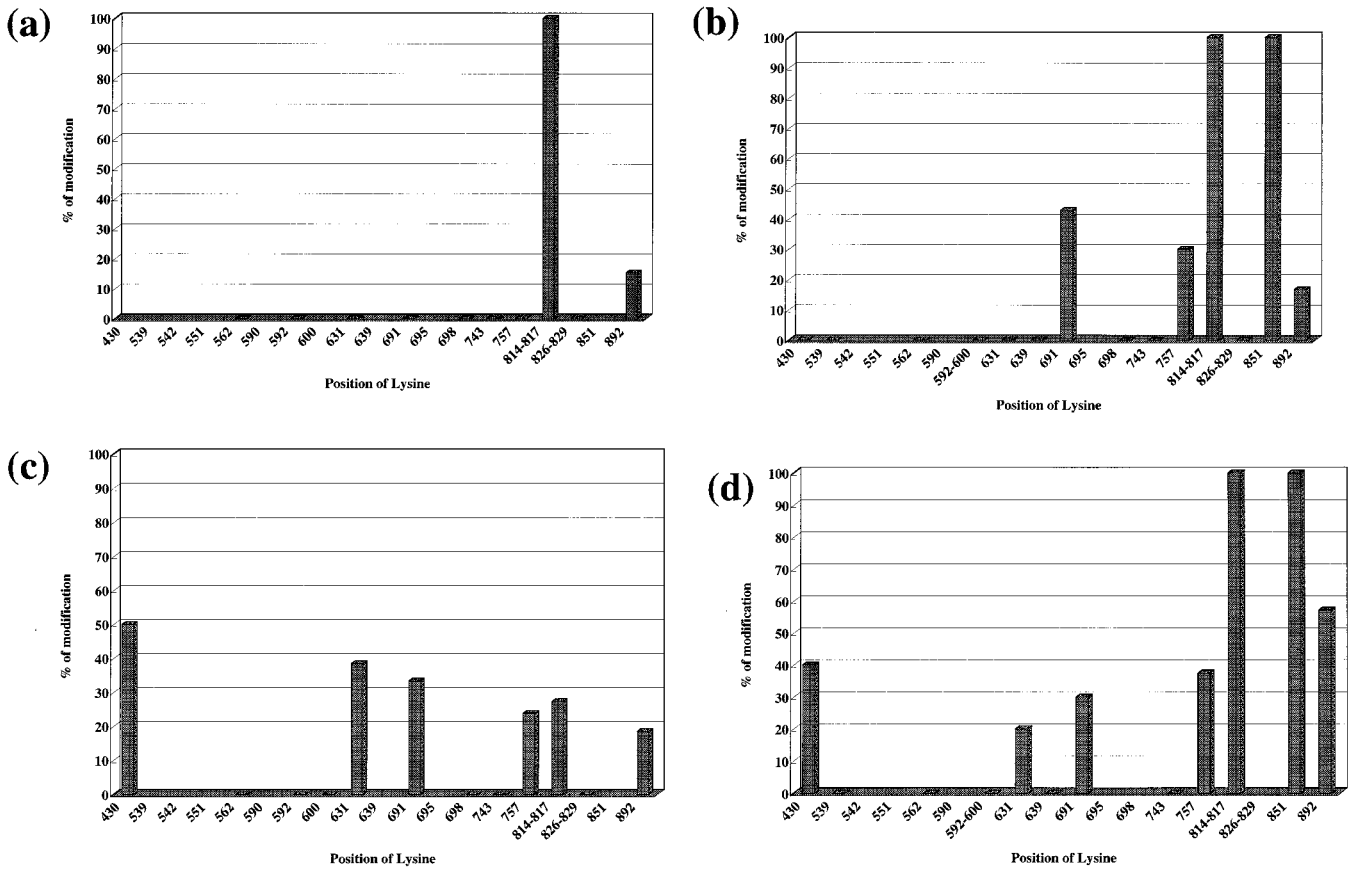

FIGURE 2: Modification of the available lysine residues of band 3 in normal and SAO membranes pretreated with 10 mM NaOH (pH 12) or with 100 mM NaOH (pH 13). Normal and SAO red cell membranes were pretreated with 10 mM NaOH (pH 12) or with 100 mM (pH 13) and modified with 0.25 mM NHS-SS-biotin in 50 mM sodium bicarbonate buffer (pH 8.5) at 4 °C for 30 min. In the case of the membranes pretreated with 10 mM NaOH and chemically modified, we further treated the membranes with 100 mM NaOH. The alkali-treated, NHS-SS-biotin-modified membranes were digested with chymotrypsin and trypsin, and the digested membranes were solubilized with an equal volume of 4 M urea/2% SDS. The peptides in the solubilized membranes were separated by HPLC using a reversed-phase column (Cosmosil 5C18-AR-300 or Cosmosil 5C18-MS, 4.6 × 250 mm; Chemco, Osaka, Japan), with a water/acetonitrile/propanol eluant system containing 0.1% trifluoroacetic acid as described under Experimental Procedures. Fractions were collected every 2 min, and all fractions were analyzed using a gas-phase sequencer (Applied Biosystems, model 492). The phenylthiohydantoins were identified with an Applied Biosystems 140C on-line phenylthiohydantoin analyzer system. All fractions were also analyzed by MALDI-TOF mass spectrometry using a Voyager RP with BioSpectrometry workstation (PerSeptive Biosystems, Framingham, MA). The fraction of each of the lysine-containing peptides in the band 3 membrane domain modified by biotinylation was determined and is expressed a percentage of the total peptide (modified plus unmodified). (a) Modification of lysine residues of band 3 in normal membranes pretreated with 10 mM NaOH. (b) Modification of lysine residues of band 3 in SAO membranes pretreated with 10 mM NaOH. (c) Modification of lysine residues of band 3 in normal membranes pretreated with 100 mM NaOH. (d) Modification of lysine residues of band 3 in SAO membranes pretreated with 100 mM NaOH.

samples pretreated with 10 mM NaOH (Table 1). Although the overall recovery of peptides originating from some cleavage sites was low, the recoveries of fragments from the normal and from the SAO sample were similar for the cleavages at Phe-379, Phe-511, Arg-514, Tyr-553, Leu-886, and Lys-892. As expected, the major site of cleavage of normal band 3 was at Leu-558, in the extracellular loop 5−6 that is susceptible in red cells. This was also the major site of cleavage of band 3 in SAO membranes, but there was a reduction in cleavage yield by about 50%. A marked reduction in cleavage yield was also observed for the cleavages at Arg-384, Arg-387, Lys-551, Lys-695, Lys-698, Trp-848, Leu-857, and Arg-901. It is likely that essentially only the normal band 3 in the SAO membranes was being cleaved at these sites. In addition, two sites (Phe-537 and Leu-540) were only cleaved in the SAO membranes. Surprisingly, Arg-432 was cleaved only in the normal membranes and not in the SAO membranes, even though normal band 3 makes up more than half the band 3 in the SAO membranes.

Proteolysis of Normal and SAO Red Cell Membranes Treated with 100 mM NaOH. Many sites in band 3 were cleaved to equal extents in the normal and in the SAO membranes pretreated with 100 mM NaOH and modified with NHS-SS-biotin (Tables 1 and 2). However, several band 3 sites were cleaved in the normal membranes that were not cleaved in the SAO membranes (which contain a mixture of normal and SAO band 3). Conversely, some sites were cleaved in the band 3 mixture present in the SAO membranes that were not cleaved in the normal sample. It seems unlikely that the differences in the band 3 cleavage patterns of the normal and SAO membranes result from incomplete proteolysis, since many other band 3 sites were cleaved at a similar level in the two samples. The different cleavage patterns most likely reflect differences in structure between the band 3 in the normal and SAO membrane preparations.

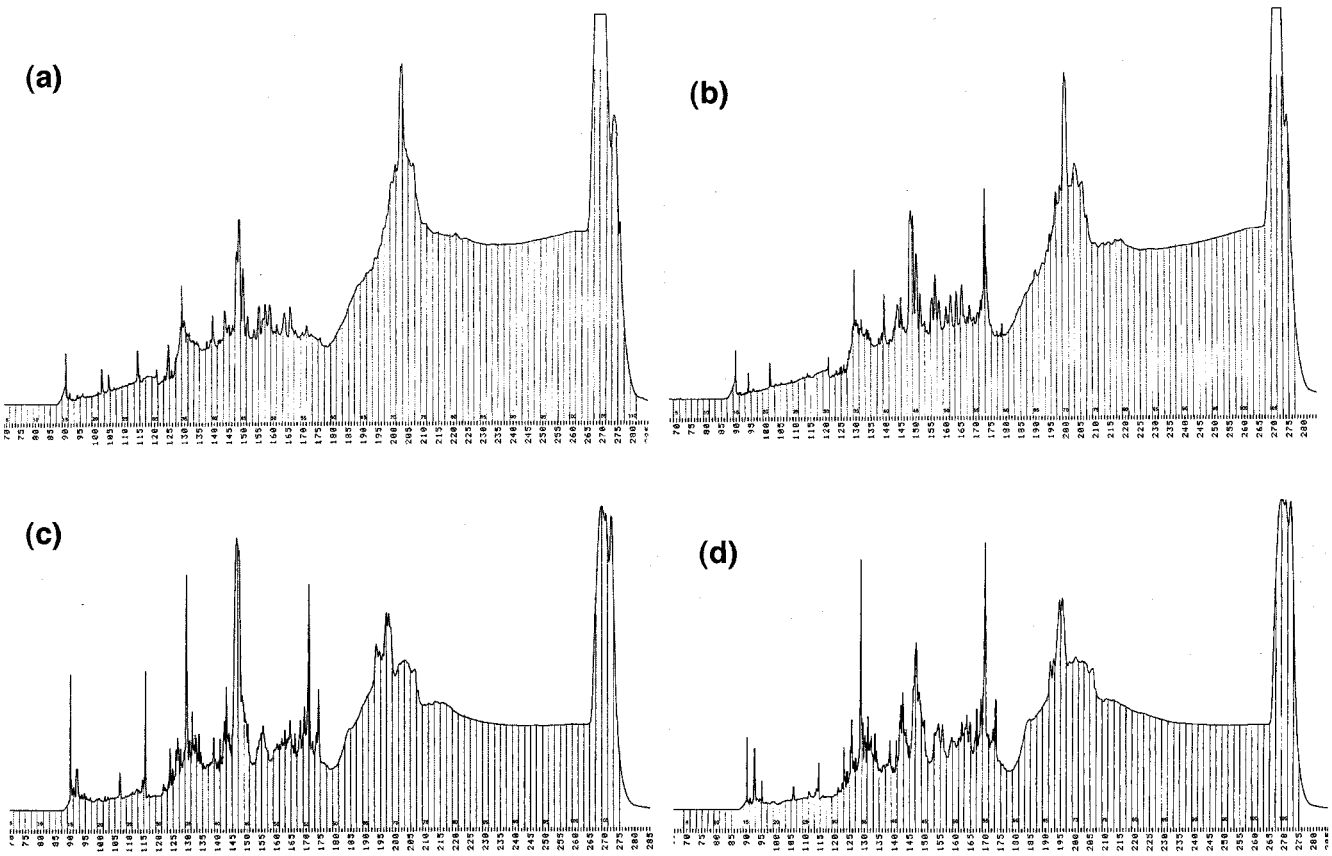

FIGURE 3: HPLC separation profiles of the peptide fragments obtained after proteolytic digestion of normal and SAO red cell membranes modified with NHS-SS-biotin. Normal and SAO membranes were pretreated with 10 mM NaOH (pH 12), or with 100 mM (pH 13), and modified with 0.25 mM NHS-SS-biotin in 50 mM sodium bicarbonate buffer (pH 8.5) at 4 °C for 30 min. The membranes that were pretreated with 10 mM NaOH and chemically modified were further treated with 100 mM NaOH. The membranes that were pretreated with 100 mM NaOH and chemically modified were digested without further treatment. The alkali-treated and NHS-SS-biotin-modified membranes were digested with chymotrypsin and trypsin, and the digested membranes were solubilized with an equal volume of 4 M urea/2% SDS. Peptides in the solubilized membranes were separated by HPLC and analyzed as described in the legend to Figure 2. The absorbance profiles at 215 nm are shown. (a) HPLC separation profiles of peptide fragments in digested normal membranes pretreated with 10 mM NaOH and chemically modified. (b) HPLC separation profiles of peptide fragments in digested SAO membranes pretreated with 10 mM NaOH and chemically modified. (c) HPLC separation profiles of peptide fragments in digested normal membranes pretreated with 100 mM NaOH and chemically modified. (d) HPLC separation profiles of peptide fragments in digested SAO membranes pretreated with 100 mM NaOH and chemically modified.

DISCUSSION

The Structure of Band 3 in Normal Membranes. Only two lysine residues in band 3 were modified by NHS-SS-biotin in normal membranes treated with 10 mM NaOH. Lys-814/Lys-817 was completely biotinylated, while Lys-892 in the C-terminal tail of the protein was partially reactive (Figures 2a and 4a). A synthetic peptide containing the region of Lys-814 and Lys-817 had a solution structure comprising two helices joined by a mobile loop with these residues located at the apex of the loop (*27*). This loop was suggested to form the end of the large protrusion extending from the membrane which is evident in the low-resolution three-dimensional map of the band 3 membrane domain (*20*). The complete reaction of Lys-814/Lys-817 with NHS-SS-biotin probably reflects its exposed position in the protein.

Band 3 in normal membranes treated with 10 mM NaOH was cleaved at few sites by sequential proteolysis using chymotrypsin and trypsin. As expected (*23*, *24*), much more extensive cleavage was obtained after membranes were treated with 100 mM NaOH (Tables 1 and 2 and Figure 4a). Most protease cleavage sites in both band 3 preparations occur near the boundaries of transmembrane segments or in surface loops. However, as reported previously (*6*, *24*), some protease cleavages occur within the regions containing TM1−3, TM6, TM8−10, and TM13−14 of band 3 (Figure 4a), which are predicted to be membrane-embedded in the 14 span model. These regions probably interact primarily with other peptide portions and are shielded from the membrane lipids (*6*, *24*). They are extruded from the membrane bilayer under denaturing conditions and become accessible to proteases.

SAO Band 3 Alters the Structure of Normal Band 3 in SAO Red Cell Membranes. Both the proteolysis experiments and the biotinylation studies show that the normal band 3 in SAO membranes has changed structure, as is evident from the data summarized in Table 2 and Figure 4. These data show that many band 3 cleavages observed in normal membranes treated with 100 mM NaOH are not observed in 100 mM NaOH treated SAO membranes, a surprising observation given that normal band 3 comprises more than half the band 3 in SAO membranes. The results demonstrate that band 3 SAO interacts directly or indirectly with essentially all the normal band 3 in SAO membranes and

Table 1: Recovery of Peptides from Each Cleavage Site in Band 3 of Normal and SAO Membranes Treated with 10 mM NaOH or 100 mM NaOH

	recoveries of peptide fragments (pmol) in					recoveries of peptide fragments (pmol) in			
	10 mM NaOH		100 mM NaOH			10 mM NaOH		100 mM NaOH	
cleavage site	normal	SAO	normal	SAO	cleavage site	normal	SAO	normal	SAO
original pmol	2500	2500	2500	2500	655L			400	
379F	180	150	1500	1200	656R			600	500
384R	500	60			659F			400	
387R	150	60		350	679F			150	
389R			70	50	687L			350	350
392Y				50	694R			350	350
394L			70		695K	80	50	200	100
401F			400		698K	80	50	200	100
423F			400	150	702F			280	300
430K				150	706L			100	
432R	40		550	200	719F			180	380
464F			350	200	730R			650	400
471F			200		743K			1000	1100
476F			200	60	754Q				100
507F			200	60	757K			1000	700
511F	50	40	400	200	760R				300
514R	50	40	400	200	782R			140	50
519Y			250		811L			250	
534Y				150	813F				80
537F		30	150		818Y				80
540L		30		150	820P			100	
551K	1100	460	1600	1100	824Y			400	400
553Y	150	150			827R			330	
558L	1250	610	1600	1100	831W			200	80
562K			80	200	834H			330	
584F			830	250	836F			600	480
590K			750		848W	30	10	300	150
591F				100	851K			250	
596Y			750		857L	30	10		150
600K				100	871R			250	
602R			250		878F			1100	900
623F			250		879R			80	
628Y			450	250	884Q			200	80
632L			450	250	886L	100	100	300	100
639K				250	892K	100	100	1400	900
648W			1600	500	895F			200	180
653L			600		901R	250	100		400

therefore alters its structure or its accessibility to protease cleavage. The observation that Lys-851 (which is not biotinylated in normal membranes) is 100% modified by the biotinylation reagent in SAO membranes reinforces this view. We infer from these data that the band 3 in SAO membranes is not associated as dimers but is present predominantly as tetramers and higher oligomers. As a result, the fraction of normal band 3 present as monomers, homodimers, or higher homooligomers in SAO membranes is too low to be detected by our methodology. Other evidence also suggests the band 3 in SAO membranes has a greater tendency to aggregate than normal band 3 and greater propensity to form tetramers (*16*, *18*). Freeze−fracture electron micrographs of SAO red cells also show the presence of unusual linear microaggregates of intramembranous particles that most likely contain band 3 (*32*).

Table 2 and Figure 4 show that the normal band 3 sites not cleaved in band 3 of SAO membranes are distributed over the entire band 3 molecule. Clearly, the structural change in band 3 SAO is transmitted throughout the entire membrane domain of the normal band 3 in SAO membranes. The resistance of the normal band 3 sites to proteolysis in SAO membranes could result from their occlusion by interaction with band 3 SAO or structural changes that alter local conformation or local resistance to NaOH denaturation. These widespread structural changes induced in both the normal and the SAO protein by the SAO deletion are to be expected if the band 3 oligomer is a close-packed assembly of TM regions with extensive interactions across the monomer interfaces, and if the structure has considerable flexibility. The structural flexibility of band 3 has been noted previously, since the protein can accommodate the presence of several overlapping TM spans and still retain functional activity (*21*). Perturbations induced by the SAO mutation within this close-packed structure would be transmitted throughout all regions of the oligomer.

Intramolecular and Intermolecular Interactions in Band 3. Previous studies suggest that the membrane domain of band 3 contains subdomains consisting of TM1−5, TM6−8, and TM9−10 (*6*, *24*). Support for this model also comes from observations that the correct assembly of TM segments within these subdomains required interactions with neighboring spans (*28*−*31*). Experiments on the association and coimmunoprecipitation of biosynthetic fragments of band 3 also suggest that TM1−5 and TM9−12 form subdomains (*21*).

The presence of novel protease cleavage sites in the TM1−5 region in SAO membranes (Table 2) confirms other data that the SAO deletion causes misfolding that extends

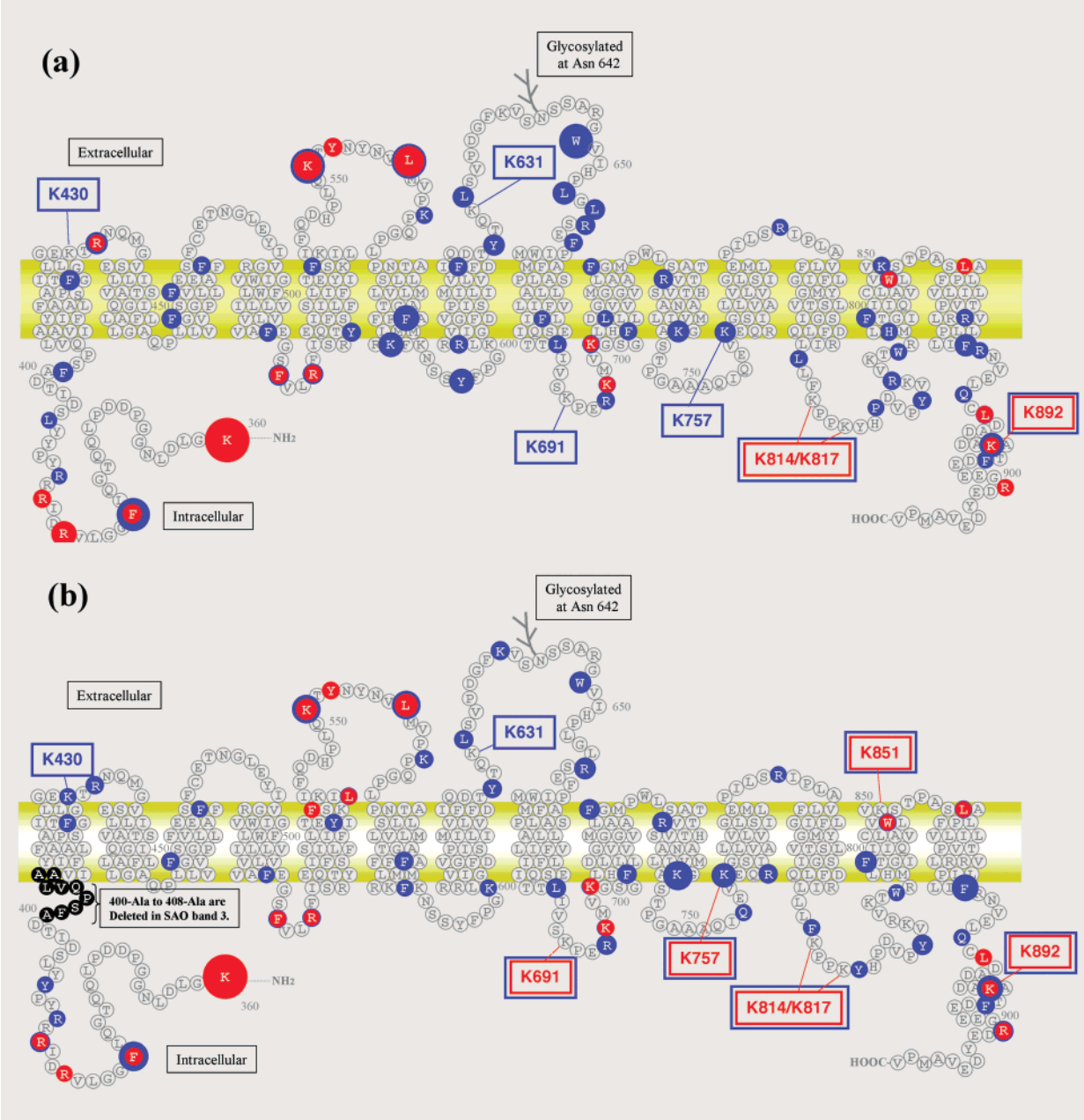

FIGURE 4: Location of the chemically modified lysine residues and proteolytic cleavage sites of band 3 in normal and SAO membranes based on the 14 span model for band 3 topology. A 14 span model (*8*) for the folding of band 3 is used to indicate the location of the sites modified by NHS-SS-biotin (residue numbers shown in boxes) and the sites of proteolytic cleavage (shown by filled circles). The red boxes and red circles show the sites modified by biotinylation and the sites of proteolytic cleavage in membranes treated with 10 mM NaOH, while the blue boxes and blue circles show the sites modified by biotinylation and the sites of proteolytic cleavage in membranes treated with 100 mM NaOH. Sites that underwent proteolytic cleavage or biotinylation in both 10 mM NaOH and 100 mM NaOH-treated membranes contain both colors. The sizes of the filled circles give an approximate indication of the relative amounts of proteolytic cleavage at each site. (a) Biotinylation sites and proteolytic cleavages in band 3 of normal red cell membranes. (b) Biotinylation sites and proteolytic cleavages in band 3 of SAO red cell membranes.

throughout this subdomain (*12*, *15*, *16*). The absence, in SAO membranes, of several normal band 3 cleavage sites in this region (Table 2) indicates that TM1−5 of normal band 3 in SAO membranes also has abnormal structure, and is consistent with the presence of extensive interactions between the TM1−5 subdomains of different band 3 monomers within the band 3 oligomer.

The complete biotinylation in SAO membranes of the normally unreactive Lys-851 (located in the extracellular loop 13−14) is very striking (Figure 2). The effects of the SAO deletion (located at the cytoplasmic end of TM1) are known to extend to the extracellular surface and abolish the reactivity of Lys-430 (in the extracellular loop 1−2) toward eosin maleimide (*16*). This suggests that the altered reactivity of Lys-851 results from the perturbation of interactions between the extracellular loops 1−2 and 13−14 induced by the SAO deletion. Since this reactivity is changed in both types of band 3 in SAO membranes, this altered interaction is also transmitted across the interfaces between hetero-oligomers to the normal band 3 monomers. These changes in both band 3 types in SAO membranes are confirmed by the proteolysis studies that suggest the changes extend over the entire

Table 2: Band 3 Proteolysis Sites in SAO and Normal Membranes Treated with 100 mM NaOH

only in SAO	similar in SAO and normal	normal sites cleaved at reduced level in SAO	only in normal	predicted location[a]
387R	379F		394L	N-terminal
392Y	389R		401F	
		423F		**TM1**
430K		432R		**loop 1−2**
		464F		**TM3**
		476F	471F	
		507F		**loop 4−5/TM4 junction**
		511F		**loop 4−5**
		514R		
534Y			519Y	**TM5**
540L			537F	
	551K			loop 5−6
	558L			
		584F	590K	**TM6**
591F				loop 6−7
600K			596Y	
			602R	loop 6−7/TM7 junction
			623F	**loop 7−8/TM7 junction**
639K		628Y		**loop 7−8**
		632L		
		648W	653L	
	656R		655L	
			659F	
	687L		679F	**TM8**
	694R			
		695K		loop 8−9
		698K		
	702F		706L	**TM9**
	730R			TM10
	743K			**loop 10−11**
754Q	757K			
760R				**TM11**
		782R		loop 11−12
813F			811L	**loop 12−13**
818Y	824Y		820P	
		831W	827R	
			834H	**TM13**
	836F	848W	851K	
857L				**TM14**
	878F		871R	C-terminal
	892K	884Q	879R	
901R	895F	886L		

[a] Predicted locations are based on the 14 TM span model of band 3 presented by Wood (*8*). The cleavage sites listed in the columns headed "normal sites cleaved at reduced level in SAO" and "only in normal" originate from the normal band 3 in the normal and SAO membrane preparations (see text). The boldface areas highlight regions where the sites of protease cleavage and conformation of band 3 differ in normal and SAO membranes.

TM13−14 region. The differences in protease cleavage sites extend from the cytoplasmic loop 12−13 to the C-terminus of the protein (Table 2, Figure 4). This suggests that the TM13−14 region may interact directly with the TM1−5 subdomain. It has been shown that Lys-539 (at the extracellular end of TM5) and Lys-851 (in the 13−14 loop) can be cross-linked by H_2DIDS within the same band 3 monomer (*17, 33*), indicating that these two lysine residues are separated by approximately 15 Å. This distance (about 1.5 helix diameters) would also be consistent with the juxtaposition of the TM1−5 domain and the very small 13−14 loop. The structural changes in the TM1−5 domains of the band 3 SAO monomers are probably transmitted to the TM13−14 regions of associated normal band 3 molecules through the interaction between TM1−5 subdomains as well as through other regions of the oligomer interfaces.

Interactions at the Surfaces of Band 3. The SAO deletion also alters the structure in the TM8−9 portion of the normal protein in SAO membranes. This is evident from the many changes in the protease cleavage sites in the large *N*-glycan-containing extracellular loop 7−8 and in TM8 and TM9 (Table 2, Figure 4). The cluster of changes in loop 7−8 suggests this region may also interact with the extracellular surface of the TM1−5 subdomain. Although TM8 has been suggested to be located at the periphery of band 3 (*21*), the large size of loop 7−8 could allow this interaction.

Lys-691 and Lys-757 are unreactive to biotinylation in normal membranes treated with 10 mM NaOH (Figure 2a) but become reactive to biotinylation in SAO membranes treated with 10 mM NaOH (Figure 2b) to a similar extent as in normal membranes denatured with 100 mM NaOH (Figure 2c). This suggests that the loops containing Lys-691 (loop 8−9) and Lys-757 (loop 10−11) of band 3 in SAO membranes are destabilized compared to the normal protein, and become susceptible to denaturation at a lower NaOH concentration than normal band 3. The modification of these residues is similar to that observed with normal membranes treated with 100 mM NaOH (compare Figure 2b and Figure 2c), indicating that the destabilization of these regions involves both the normal and SAO band 3 populations. The disruption by the SAO deletion of the structural subdomain at the cytoplasmic boundary of TM1 (*15*) therefore affects the stability of the cytoplasmic loops 8−9 and 10−11, suggesting these three regions interact at the cytoplasmic surface within the SAO monomer and in the hetero-oligomers of normal and SAO band 3. The presence of a cluster of cryptic normal band 3 protease cleavage sites in the cytoplasmic loop 12−13 (Table 2, Figure 4) suggests this loop may also interact with the cytoplasmic surface of the TM1−5 subdomain.

In summary, the SAO deletion causes misfolding of the TM1−5 domain, and misfolding of this domain perturbs the structure of other regions of band 3 which normally interact with it. These include the TM13−14 region, the extracellular 7−8 and 13−14 loops, and the cytoplasmic 8−9, 10−11, and 12−13 loops. These structural perturbations are transmitted from SAO band 3 to the normal band 3 in SAO membranes because the presence of SAO band 3 causes the formation of hetero-tetramers or higher hetero-oligomers.

The Dominant Effect of Band 3 SAO on the Structure of the Other Band 3 Alleles in SAO Heterozygotes Has Functional Consequences in Red Cells and in Band 3-Associated Disease. The changes induced by band 3 SAO on the structure of the other band 3 alleles in SAO heterozygotes account for the significant functional alterations that have been reported in the red cells of these SAO heterozygotes. These changes include the following: (a) altered anion transport activity of the allelic band 3 partner; (b) increased red cell rigidity in compound heterozygotes; and (c) anemia in compound heterozygotes.

Sulfate and chloride transport studies on the red cells of simple SAO heterozygotes show that although normal band 3 comprises 55% of the band 3 in SAO red cells, these cells have only 42−43% of the anion transport activity of normal red cells (*12, 14*). The transport-inactive band 3 SAO

therefore decreases the transport activity of the normal band 3 to 75% of its activity in normal cells.

An N-terminally truncated form of red cell band 3 is involved in distal renal tubular acid secretion (*34*, *35*). Several band 3 mutations have been associated with familial distal renal tubular acidosis (dRTA; reviewed in ref *7*). Three of these mutant band 3 types (Gly701 → Asp [G701D], Ala858 → Asp [A858D], and the deletion of Val-850 [ΔV850]) have been found in association with band 3 SAO in Malaysia, Thailand, and Papua New Guinea (*14*, *36*). The red cells of the A858D and ΔV850 mutants had almost normal specific anion transport activity when present as heterozygotes with normal band 3; however, the specific anion transport activity of these two mutant proteins was substantially reduced (to 55% and 17%, respectively, of the activity of the normal protein) in compound heterozygotes with band 3 SAO (*14*). Coexpression studies in *Xenopus* oocytes suggest that band 3 SAO also decreases the enhancement of the cell surface movement of the band 3 mutants to the cell surface by glycophorin A (*14*).

It is well-known that SAO red cells are more rigid than normal red cells (*10*, *37*). The red cells of simple heterozygotes with the G701D or A858D mutations had normal red cell rigidity. However, compound heterozygotes of G701D or A858D with band 3 SAO had membrane rigidity that was markedly higher than even that of the simple SAO heterozygotes in the same families (*14*).

Family studies also indicate that anemia is present in compound heterozygotes of the G701D, A858D, or ΔV850 mutations with band 3 SAO, while simple heterozygotes of these dRTA-associated mutations do not have anemia (*14*). All these observations indicate that band 3 SAO also changes the structure of these mutant band 3 proteins in compound heterozygotes with deleterious effects on red cell function. Similar effects in the kidney may exacerbate the dRTA in these compound heterozygotes. Mutual structural changes in compound heterozygotes of other dRTA-associated band 3 mutations such as A858D/ΔV850 may also be responsible for the anemia and severe complications of dRTA observed in these individuals (*14*).

The selective chemical modification and proteolytic cleavage methods used in this work provide sensitive tools for studying changes in the structure of multispanning membrane proteins which result from mutations. Our results clearly show that the presence of SAO band 3 has major structural effects on normal band 3 or other band 3 alleles in the red cell membranes of SAO heterozygotes. These dominant structural effects can compromise red cell function. The transmission of structural perturbations by intra-oligomer interactions is likely to be a general mechanism through which a mutant allele of an oligomeric multispanning membrane protein can exert dominant effects on the function and other properties of the other allele.

SUPPORTING INFORMATION AVAILABLE

Supplementary Tables 1−6 includes detailed information on the characterization and recovery of the peptide sequences obtained from each of the proteolytic digestion experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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