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Cloning and characterization of band 3, the human erythrocyte anion-exchange protein (AE1)

(anion antiporter/DNA cloning/erythrocyte membrane)

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ABSTRACT **The human erythrocyte anion-exchange protein (band 3 or AE1) was cloned from a fetal liver cDNA library. Three overlapping clones, encompassing 3637 nucleotides, were analyzed in detail. These encode a 911-amino acid protein (M_r 101,791) and detect a single 4.7-kilobase species in human reticulocyte RNA. The corresponding gene is located on chromosome 17. The protein is similar in structure to other anion exchangers and is divided into three regions: a hydrophilic, cytoplasmic domain that interacts with a variety of membrane and cytoplasmic proteins (residues 1–403); a hydrophobic, transmembrane domain that forms the anion antiporter (residues 404–882); and an acidic, C-terminal domain of unknown function (residues 883–911). The N-terminal domain contains several conserved sections (e.g., residues 57–86, 102–164, 219–347, and 375–403), some of which may contribute to binding sites for ankyrin, protein 4.1, or protein 4.2. The membrane domain is highly conserved with the exception of a single segment (residues 543–567) that contains several sites for cleavage of the protein by extracellular proteases. Based on hydropathy analyses and the wealth of available topographical and functional data, a model is proposed in which the protein crosses the membrane 14 times.**

Band 3 (anion exchanger 1 or AE1)¶ is the major protein of the human erythrocyte membrane and one of the best studied of all membrane proteins. It is a chimeric molecule composed of two dissimilar and functionally distinct domains (1). The N-terminal portion forms a 43-kDa water-soluble cytoplasmic domain that has multiple binding functions, while the C-terminal transmembrane region is the physiologically important anion exchanger. Since the initial publication of mouse erythrocyte band 3 (AE1) in 1985 (1), the sequence of chicken erythrocyte AE1 (2, 3), the sequences of truncated renal forms of mouse and rat AE1 (4, 5), and the sequences of human (7) and mouse (8) band 3-related protein (AE2) have appeared. However, almost all functional and topographical studies of band 3 have been performed with proteins from human erythrocytes. In this paper we report the cloning and sequencing of a cDNA for human erythrocyte band 3.‖ Following completion of this work, Tanner *et al.* reported similar studies (6).

MATERIALS AND METHODS

cDNA Cloning. ^{32}P-labeled cDNA probes were prepared by the random priming method (10) and used to screen human fetal liver λgt11 cDNA libraries that had been transferred to duplicate nylon filters (Biotrans, 1.2 μm; ICN) by standard plaque-lift procedures and fixed by UV bonding. The filters were prehybridized, hybridized, and washed as described (8). DNA was prepared from plate lysates of plaque-purified bacteriophage DNA and was subcloned into Bluescribe and Bluescript-KS(+) plasmid vectors (Stratagene) and amplified by standard procedures (11).

cDNA Sequencing. Selected clones were sequenced either by "shotgun" (12) or exonuclease III/S1 nuclease deletion (13) methods. The deletion method was modified so that deletion plasmids of the desired size were isolated by electrophoresis in low-melting-point agarose, religated in agarose, and used to transform *Escherichia coli* NM522. Double-stranded plasmid templates were prepared by the method of Kraft *et al.* (14). In both cases sequencing reactions were performed by the dideoxy method as modified by Biggin *et al.* (15) and Bankier (16), using, as needed, Klenow or modified T7 DNA polymerase (Sequenase; United States Biochemical) for elongation reactions. 7-Deaza-GTP was substituted for GTP as needed for refractory sequences. Sequence data were compiled and analyzed using the University of Wisconsin Genetics Computer Group (UWGCG) programs.

Chromosomal Location of Band 3 Gene. The *Eco*RV–*Xba* I fragment of pHB3-22 was labeled with [α-^{32}P]dCTP (10) and hybridized to human chromosomes separated by fluorescence-activated cell sorting (17).

RESULTS AND DISCUSSION

Band 3 Cloning. Clones were obtained from two different fetal liver cDNA libraries. Both libraries were prepared from livers obtained at a stage in gestation (≈20 weeks) where the liver contains large numbers of erythroid precursors. The initial library, in the plasmid pKT218 (a gift of S. Orkin, Harvard Medical School), was screened with a full-length cDNA (pB3SP4) to murine erythrocyte band 3 (1) and yielded a single positive clone (pHB3-HFL9; Fig. 1*A*). Because no further clones were obtained on rescreening, a larger fetal liver cDNA library (2×10^7 recombinants; gift of B. Forget, Yale University School of Medicine) was examined using the 915-bp *Pst* I fragment of pHB3-HFL9 and the 5′ end of one of the recovered clones (pHB3-9) as probes. The 3 largest clones of the 45 detected, pHB3-9 [1.94 kilobases (kb)], pHB3-22 (2.48 kb) and pHB3-45A/B (3.13 kb), were chosen for further characterization.

Size of Band 3 mRNA. Northern blot hybridization of human reticulocyte total and poly(A)-selected RNA, using pHB3-HFL9 as a probe, showed a single band of 4.7 kb (data

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Abbreviations: G3PD, glyceraldehyde-3-phosphate dehydrogenase; H_2DIDS, 4,4′-diisothiocyanato-1,2-diphenylethane-2,2′-disulfonate.

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¶By convention AE1 (anion exchanger 1), AE2, and AE3 refer to the products of the AE1 gene (band 3) (1–6), AE2 gene (band 3-related protein) (7, 8), and AE3 gene (brain anion exchanger) (9), respectively.

‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M27819).

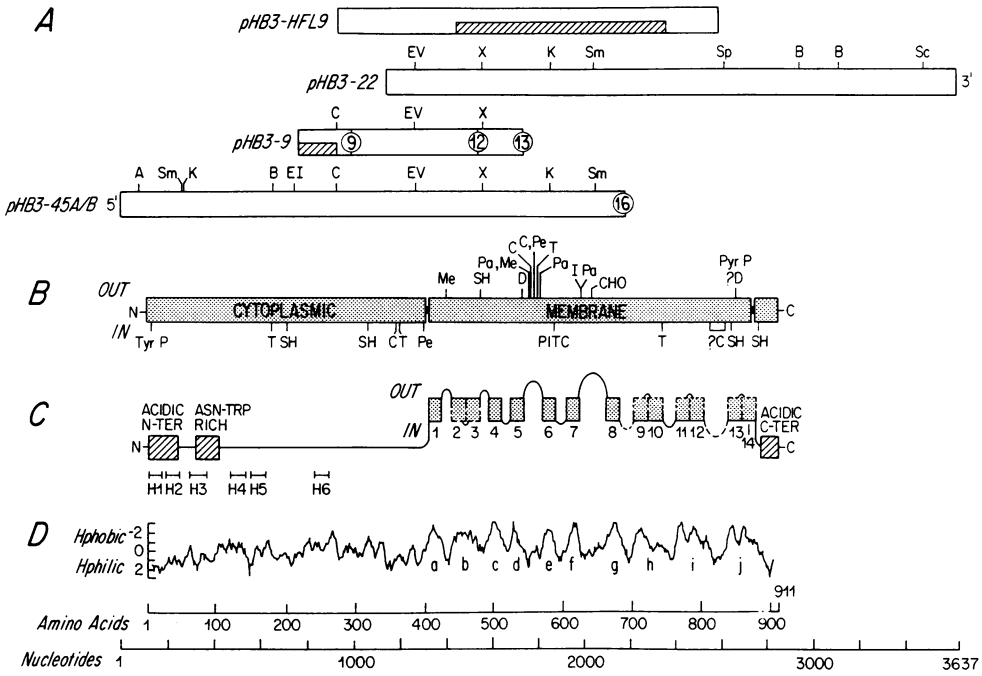

FIG. 1. Organization of human band 3 and its cDNA clones. (*A*) Organization of cDNA clones (pHB3-HFL9, pHB3-9, pHB3-22, pHB3-45A/B) with respect to one another. Restriction sites: A, *Acc* I; B, *Bam*HI; C, *Cla* I; EI, *Eco*RI; EV, *Eco*RV; K, *Kpn* I; Sc, *Sac* I; Sm, *Sma* I; Sp, *Sph* I; X, *Xba* I. Encircled numbers indicate location of retained introns in cDNA clones. Relative to introns in the mouse band 3 gene (18) these are as follows: 9, intron 9 (or I; GenBank designation) [539 base pairs (bp)]; 12, intron 12 (or L) (114 bp); 13, intron 13 (or M) (>318 bp); and 16, intron 16 (or P). Hatched areas: in pHB3-HFL9, the 915-bp *Pst* I fragment used to obtain pHB3-9 and pHB3-22; in pHB3-9, *Cla* I fragment used to obtain pHB3-45A/B. (*B*) Organization of band 3 protein. Sites indicated above the schematic protein are accessible from outside the membrane; those below are exposed on the cytoplasmic surface. Sites are shown for cleavage by trypsin (T) (Arg-180, Lys-360, Lys-562, Lys-743) (1, 19–21), chymotrypsin (C) (Tyr-359, Tyr-553, Leu-558, and probably between Phe-813 and Phe-836) (19, 22–24), papain (Pa) (Gln-550, Gln-564, Gln-630) (21), and pepsin (Pe) (Asp-396, Leu-558) (20, 25). Sites of tyrosine phosphorylation (TyrP) (Tyr-8 and probably Tyr-21) (26, 27), exofacial lysine methylation (Me) (Lys-430, Lys-551) (28), external iodination (I) (Tyr-628) (20), and glycosylation (CHO) (Asn-642) (21, 22, 29) are also designated. Lysines that are covalently modified by the anion-transport inhibitors 4,4′-diisothiocyanato-1,2-diphenylethane-2,2′-disulfonate [H_2DIDS (D)] (Lys-539 or Lys-542 and possibly Lys-851) (30, 31), phenylisothiocyanate (PITC) (Lys-590) (20), and pyridoxal phosphate (PyrP) (Lys-851) (31) are noted. Cysteines (SH) are present at positions 201, 317, 479, 843, and 885. All are accessible from the cytoplasm (24, 32) except Cys-479, which can probably be labeled from the exterior with some reagents (33, 34). There is evidence that Cys-843 may normally be covalently modified (31). (*C*) Structural features within the cytoplasmic domain include an acidic N-terminus, a segment rich in asparagine and tryptophan, and six regions (H1–H6) that are predicted to be α-helical. The locations of 14 putative membrane-spanning regions are shown by stippled boxes. These regions are joined by segments that loop up (exterior) or down (cytoplasm). Lines enclosing adjacent transmembrane segments 2/3, 9/10, 11/12, and 13/14 are partially dashed to indicate uncertainty as to whether the polypeptide chain passes partly or completely through the lipid bilayer before reversing direction. Loops 8–9 and 12–13 are dashed to indicate that their location is less certain than the rest. (*D*) Hydropathy plot of the deduced amino acid sequence, obtained with the algorithm and hydropathy values of Kyte and Doolittle (35) and a window of 11 amino acids. The 10 hydrophobic regions are designated a–j. The 14 predicted transmembrane regions are shown above in *C*. All materials in *A–D* are aligned and are drawn to the same relative scale.

not shown). This is slightly larger than the murine band 3 message (4.3 kb) (1, 7). As expected for erythroid band 3 (7), no hybridizing transcripts were detected in RNA from either induced or uninduced K562 erythroleukemia cells.

Location of the Band 3 Gene. Hybridization of the *Eco*RV–*Xba* I fragment of pHB3-22 to isolated human chromosomes localized the human band 3 gene to chromosome 17, in agreement with the finding of Showe *et al.* (36).

Nucleotide Sequence. The combined clones extend 114 bases upstream from the translation start site and 790 bases beyond the C-terminal amino acid codon, a total distance of 3637 bases. However, one or both of the noncoding regions are incomplete, since pHB3-22 does not contain a poly(A) tail or polyadenylylation signal, and since, as noted above, the full-length mRNA is ≈4.7 kb long.

Clones pHB3-9 and pHB3-22 were completely sequenced on both strands. Clone pHB3-45A/B was also sequenced on both strands through the *Cla* I site (Fig. 1*A*) and on one strand from there to the end of its coding region (base 2171). The overlapping regions of the three clones were identical. Bases 115–2847 form a long open reading frame encoding a 911-amino acid polypeptide of 101,791 Da (Fig. 2). The predicted translation initiation site conforms closely to the Kozak consensus sequence (37) and matches the previously published N-terminal sequence (38). The predicted C-terminal valine agrees with earlier work (22, 39) and corresponds closely to the C-terminal sequence of mouse erythrocyte band 3 (1).

Interestingly, two of the four clones retain unspliced introns (Fig. 1*A*), which suggests that the kinetics of splicing band 3 transcripts may be unusual. All four human band 3 introns were found at known locations of mouse band 3 introns (18) and were bounded by typical splice donor and acceptor sequences.

Comparison with Previously Published Portions of the Human Band 3 Amino Acid Sequence. The translated amino acid sequence of band 3 (Fig. 2) corresponds closely with reported sequence fragments (19–22, 25, 31, 38) with a few exceptions. (*i*) The reported aspartic acid at position 11 (38) is a methionine in our sequence. (*ii*) We do not detect the second glutamic acid at position 69 (38). (*iii*) The phenylalanine observed at residue 425 (25) is a glycine. The nucleotide sequence agrees precisely with that reported by Tanner *et al.* (6) with four exceptions, one of which affects the coding sequence. We find, in agreement with Kaul (38), that residue 56 is a lysine instead of a glutamic acid (6) (AAG instead of

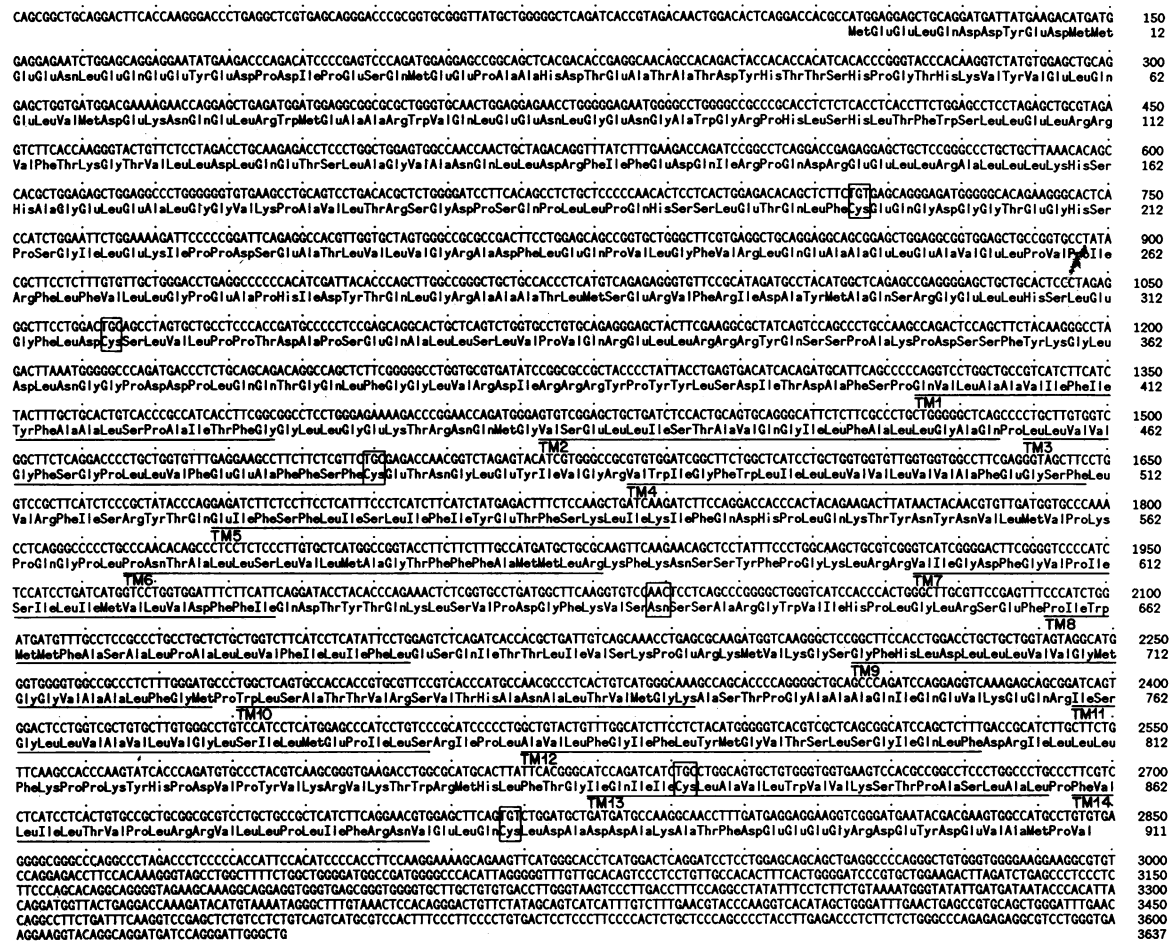

FIG. 2. Nucleotide and deduced amino acid sequence of human erythrocyte band 3 cDNA. Putative transmembrane (TM) regions are underlined. The five cysteine residues and the single glycosylation site (Asn-642) are boxed.

GAG). In the 3′ untranslated sequence we have a thymine instead of a cytosine at base 3279 (our numbering), one cytosine instead of two at base 2940, and two guanines instead of one at 3247–3248 (Fig. 2).

Cytoplasmic Domain. The cytoplasmic domain is defined as the N-terminal 403 amino acids, the point at which the first transmembrane segment is predicted to begin (Figs. 1 and 2). The majority of this segment can be released as two peptides (41 kDa and 43 kDa) when band 3 is treated with either trypsin or chymotrypsin. One of these peptides is known to result from cleavage at either Tyr-359 (chymotrypsin) or Lys-360 (trypsin) (19). The nearby Arg^{344}-Arg-Arg-Tyr^{347} and Arg^{387}-Arg-Arg-Tyr-Pro-Tyr-Tyr^{393} sequences are good candidates for the second site. Cleavage by pepsin at Asp-396 also occurs (25). This is the most distal residue known to be within the cytoplasmic domain.

The amino acid sequence of the domain differs substantially from published sequences of mouse (1) and chicken (2, 3) band 3, and from human (7) and mouse (8) band 3-related proteins, especially the N-terminal 56 amino acids. This region is very negatively charged. The first 45 residues contain 20 acidic and no basic residues. The first 11 amino acids comprise a domain known to bind hemoglobin, hemichrome, and glyceraldehyde-3-phosphate dehydrogenase (G3PD) (40, 41), at least *in vitro*. More-distal binding sites may also exist for aldolase (42, 43) and perhaps phosphofructokinase (43). This is compatible with the structure since, with the addition of two gaps, residues 12–23 almost duplicate residues 1–10 (Fig. 2). Each of these sites contains a tyrosine (positions 8 and 21) surrounded by acidic residues [$(Asp/Glu)_2$-Tyr-Glu-Asp]. Recent data indicate that these residues are phosphorylated by an erythrocyte tyrosine kinase (26, 27) and that this abolishes enzyme binding (27). Bovine band 3 also binds G3PD. The binding peptide, which begins at about residue 50, corresponds to the human band 3 sequence between residues 20 and 40 and includes the $(Asp/Glu)_2$-Tyr-Glu-Asp sequence (44). Mouse and chicken band 3 do not bind G3PD and lack this sequence.

Structure-prediction algorithms (Chou–Fasman; ref. 45) suggest that most of the α-helical regions of the cytoplasmic domain are clustered in the first half of the domain (Fig. 1). The total predicted helical content (36%) compares closely to the measured amount (46). There are hints that the last half of the domain may also be extensively folded. Several stretches of hydrophobic amino acids are present, especially residues 258–271 and, to a lesser extent, residues 226–245 and 313–338. Presumably these are buried in a folded region. This is supported by evidence that the two cysteines in this area, Cys-201 and Cys-317, can be crosslinked to each other (47–49) indicating they are neighbors in the native molecule.

Another feature of the cytoplasmic domain is its interconversion among at least three different pH-dependent conformations (50) that differ in ankyrin binding (49). This is attributed to a flexible "hinge," which is typically assigned to the region between residues 175 and 190 (41) because this proline-rich region is accessible to cleavage by trypsin at Arg-180 (1) and is therefore presumed to be unstructured and mobile. However, the region is poorly conserved among erythroid band 3 (AE1) proteins (Table 1), including the prolines. This suggests either that conformational flexibility occurs elsewhere or that it is not as functionally important as postulated.

Table 1. Conserved sequences in band 3 proteins

Human AE1 sequences	Percent similar amino acids*		
	Mouse AE1	Chicken AE1	Mouse AE2
Cytoplasmic			
1–56†	39	23	9
57–86	73	57	60
87–101	73	27	33
102–164	84	59	57
165–218‡	61	15	17
219–247	90	62	59
248–347	78	52	48
348–374§	68	25	29
375–403	92	80	70
Membranous			
404–550	93	86	81
551–567¶	59	24	6
568–878	93	77	71
C-terminal			
879–911	79	58	52
Intramembrane segments	95	83	78
Loops (except 5–6)	92	78	67

*Sequences were optimally aligned to human band 3 (AE1) and to one another by using the UWGCG program GAP. Paired amino acids were defined as similar if they had a score ≥0.6 on the Dayoff evolutionary scale as normalized by Gribskov and Burgess (51). On this scale a perfect match has a score of 1.5.
†Hemoglobin- and enzyme-binding region.
‡Includes putative hinge region.
§Includes proteolytic cleavage sites; presumably flexible, exposed regions.
¶Transmembrane loop 5–6 (Fig. 1*C*).

Other portions of the cytoplasmic domain are relatively well conserved, including residues 57–86, 102–164, 219–347, and 375–403 (Table 1). Presumably some of these regions contribute to the ankyrin, protein 4.1, or protein 4.2 binding sites, or to the site(s) of band 3 dimerization. Previous investigators have assigned ankyrin binding to the conserved region encompassed by amino acids 102–164 (1, 2, 7, 50); however, recent studies, using a battery of antibodies directed to specific sites within the cytoplasmic domain, suggest the binding site is a complex, folded region that includes residues within segments 118–162 and 174–186, sequences near Cys-201 and on the distal side of Cys-317, and portions of the acidic N terminus (49, 52).

Membrane Domain. The membrane domain extends from residue 404 to 882 and may also include, functionally at least, the very conserved sequence 379–403 (Table 1). The hydropathy plot of this region is very similar to hydropathy plots of other bands 3 and band 3-related proteins (1–8) and shows 10 possible transmembrane regions (a–j in Fig. 1*D*), some of which (b, h, i, and j) are long enough (>40 amino acids) to span the membrane twice as α-helices. This could give a maximum of 14 transmembrane regions (underlined in Fig. 2); however, the exact number of such regions and their disposition cannot be determined with certainty, despite the fact that human erythrocyte band 3 is one of the best studied of all membrane proteins in terms of its topography. Much of our knowledge is summarized in Fig. 1 *B* and *C*. It is clear that the poorly conserved peptide sequence that loops out between transmembrane regions 5 and 6 (i.e., the 5–6 loop) must be exterior, since it is readily accessible to many forms of exofacial proteolytic cleavage and chemical modification. Also the glycosylated (29) 7–8 loop is undoubtedly exterior and the 1–2 and 3–4 loops are probably exterior, based on the studies illustrated in Fig. 1*B*.

Similarly, accessibility to phenylisothiocyanate (20) and trypsin (21) localize the 6–7 and 10–11 loops to the inner membrane surface. Loops 8–9 and 12–13 are less well defined. Presumably the former lies inside, since it follows an external loop and a probable transmembrane segment. The interior location of loop 12–13 is suggested by the finding that chymotrypsin releases an 8- to 9-kDa peptide containing both C-terminal sulfhydryl groups when it is applied to unsealed erythrocyte membrane ghosts but not to intact red cells (24). The boundaries of this peptide are unknown, but Cys-843 and Cys-885 are separated by 4.7 kDa; hence the remaining peptide must be 3.3–4.3 kDa. Depending on where chymotrypsin cleaves the peptide chain between Cys-885 and the C terminus, the proximal portion of the peptide lies somewhere between residues 804 and 839. This corresponds closely to the probable location of loop 12–13 (residues 807–838).

It is notable that, with the exception of loop 5–6, and to a lesser extent loop 7–8, all of the transmembrane segments and intervening loops are highly conserved (Table 1). This suggests that the entire transmembrane portion of band 3 is involved in its transport function and that the loops are tightly folded and interact with the transmembrane segments. This would also explain why many theoretically reactive amino acids in the loops cannot be chemically modified or proteolytically cleaved.

Amino acids that can be modified and that may be involved in anion exchange include lysine, arginine, histidine, and glutamic acid. Proximal and distal lysines in or close to the external opening of the channel are covalently modified by H_2DIDS, a specific inhibitor of anion transport (30). The proximal lysine is either Lys-539 or Lys-542 (1); Lys-539 is slightly favored since Lys-542 is missing from chicken erythroid band 3 (2, 3). Both lie near the end of transmembrane segment 5. The distal lysine is not as well localized but is functionally more important since its methylation inhibits transport, even in the absence of H_2DIDS (53). One good candidate is Lys-851. It is modified from the outside of the cell by pyridoxal phosphate, with loss of anion transport, and is protected by analogues of H_2DIDS (31, 54). Lys-851 is conserved in chicken, rat, and mouse band 3 (1–3, 5), but not in band 3-related proteins (7, 8); however, the transport properties of the latter proteins are incompletely defined.

Both intracellular and extracellular arginine residues appear to be important in anion exchange: possibly serving as critical anion binding sites. Extracellular modification of an arginine with phenylglyoxal (55) inhibits anion exchange and labels the C-terminal chymotryptic fragment discussed earlier. If loop 12–13 is inside as postulated, Arg-870 or Arg-871 would be the most accessible arginine in this fragment. Both are located in the middle of transmembrane segment 14 (Fig. 2), presumably in the anion channel. Arg-870 is the better candidate since Arg-871 is not conserved in band 3-related proteins (7, 8). If loop 12–13 is outside, as others suggest (1, 6), than Arg-808, Arg-827, and Arg-832 are possible sites. Modification of an intracellular arginine with the same reagent also inhibits transport and labels a 10-kDa pepsin fragment (55), formed by residues 559–630 (20). The likely target residues are Arg-589, Arg-602, or Arg-603.

Two externally accessible glutamate residues near the H_2DIDS binding sites can be labeled with Woodward's reagent K, with resulting inhibition of anion transport (56). One site is proximal to the external chymotryptic site at residue 553; the other is distal. Glutamates at positions 429, 439, 472, 473, 480, 485, and 535 are all candidates for the proximal site. All are conserved except Glu-480, which is missing in band 3-related proteins. Glu-535 is a particularly attractive choice because of its proximity to the putative H_2DIDS-binding Lys-539. Glu-777 is the only likely distal site in the model shown in Fig. 1*B*. It is located near the outside end of transmembrane segment 11. Band 3-related proteins

lack a glutamate at this position, but they have a neighboring aspartate (7, 8) that might be functionally equivalent.

Finally, there is some evidence that an intracellular histidine may participate in the transport process (57). The histidine residues at positions 703, 734, 819, and 834 are all conserved and potentially accessible from the inside of the membrane.

C-Terminal Domain. The last 30 amino acids or so appear to form a separate domain exposed on the inner membrane surface (58). The region is highly acidic and probably helical. Its function is unknown.

Structural Comparisons. Salhany *et al.* (54) suggested that the portions of the membrane domain proximal and distal to the exofacial chymotryptic site (Tyr-553) may form functionally equivalent but physically distinct channels. Presumably such channels would have arisen by gene duplication. If so, the duplication event is no longer apparent. Dot-matrix analyses at a variety of stringencies did not disclose the expected internal repeat (data not shown).

Similarly, no homology with other proteins was detected in extensive searches (done between March 1987 and December 1988) of the GenBank, European Molecular Biology Laboratory, and National Biomedical Research Foundation data bases, even when segments corresponding to each of the exons in mouse band 3 (18) were individually tested. In particular, we could not find a common region in comparing the cytoplasmic domain of band 3 with two other ankyrin-binding proteins: Na^+,K^+-ATPase and the Na^+ channel. A few short areas of similarity were evident in binary comparisons, such as the segment recently reported by Morrow *et al.* (59), but none of these were common to all three ankyrin-binding proteins and the homologous regions were too short and too dependent on matches between similar rather than identical amino acids to be convincing.

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