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Cross-linking and Chymotryptic Digestion of the Extracytoplasmic Domain of the Anion Exchange Channel in Intact Human Erythrocytes

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Summary. We have applied our new high yield, membrane-impermeant, protein cross-linking reagents (J.V. Staros, 1982. *Biochemistry* **21**:3950–3955) together with chymotryptic digestion of the surface of intact erythrocytes (T.L. Steck, B. Ramos, and E. Strapazon, 1976. *Biochemistry* **15**:1154–1161) in an investigation of the topology of the extracytoplasmic domain of the anion exchange channel of intact human erythrocytes. In intact erythrocytes, these cross-linking reagents have been shown to cross-link subunits of the anion exchange channel to dimers in the extracytoplasmic domain of the protein. Chymotryptic treatment of intact erythrocytes has been shown to cleave subunits of the anion exchange channel into two fragments of distinct M_r. Sequential treatment of intact erythrocytes with either of two membrane-impermeant cross-linkers, followed by digestion with chymotrypsin, yields chymotryptic fragments of the anion exchange channel cross-linked to one another. The cross-linked products observed appear to arise by cross-linking of unlike chymotryptic fragments, whether the cross-links are intersubunit or intrasubunit. These results are consistent with a model of the anion exchange channel in which the subunits form a head-to-head dimer with a twofold center of symmetry perpendicular to the plane of the membrane.

Key Words human erythrocyte · anion (exchange) channel · band 3 · cross-linking · membrane proteins

Introduction

The human erythrocyte anion exchange channel [band 3, in the nomenclature of Fairbanks, Steck & Wallach (1971)] is responsible for the rapid exchange of Cl^- and HCO_3^- across the erythrocyte membrane, which is necessary for the transport of CO_2 from the tissues to the lungs (Rothstein, Cabantchik & Knauf, 1976). This protein is a structurally important component of the erythrocyte membrane, comprising an estimated 30% of the total membrane proteins (Fairbanks et al., 1971) and forming complexes with many other membrane components including the cytoskeletal attachment protein ankyrin (Branton, Cohen & Tyler, 1981; Bennett, 1982). It also provides binding sites on the cytoplasmic face of the membrane for a number of glycolytic enzymes (Yu & Steck, 1975; Strapazon & Steck, 1977; Higashi, Richards & Uyeda, 1979).

Much effort has been invested in the chemical and physical characterization of band 3 and in the elucidation of its topological disposition in the erythrocyte membrane (*see* Steck, 1978; Knauf, 1979; Drickamer, 1980; Passow et al., 1980; Tanner, Williams & Jenkins, 1980; Rothstein & Ramjeesingh, 1980, for recent reviews). It is generally accepted that band 3 is a transmembrane protein and that the polypeptide chain of each band 3 subunit traverses the membrane several times. Further, several lines of evidence suggest that the anion exchange channel exists in the erythrocyte membrane as a dimer of band 3 subunits.

In studies of the intersubunit interactions of band 3, chemical cross-linking has played an important role (*see* Staros, Morgan & Appling, 1981, for a discussion of such experiments). Recently, we have introduced a series of membrane-impermeant cross-linkers which allow one to determine on which face of the membrane particular intersubunit interactions of membrane proteins occur (Staros et al., 1981; Staros, 1982*a*, *b*). Using our new cross-linkers, we have demonstrated that the extracytoplasmic domains of the two subunits of the band 3 dimer are in close proximity. This was first accomplished with the bis(alkylimidate) DIDIT[1] (Staros et al., 1981). Since the amidine products of the reaction of alkylimidates with primary amines retain the positive charge of the parent amino groups at physiological pH, such re-

[1] *Abbreviations used:* DIDIT, diisethionyl-3,3′-dithiobispropionimidate; BS^3, bis(sulfosuccinimidyl) suberate; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; H_2DIDS, 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid; M_r, relative molecular weight.

agents result in minimal perturbations in the structure of the protein under study; however, the relatively rapid hydrolysis of these reagents under conditions suitable for protein cross-linking results in a low yield of cross-links (Staros, 1982*a*). Our high-yield, membrane-impermeant cross-linkers BS^3 and DTSSP, while abolishing the charges of participating amino groups, result in a very much higher yield of cross-links (Staros, 1982*b*). Since we obtain apparently the same product with BS^3 and with DTSSP as with DIDIT, we have confidence that we are sampling the native structure of the anion exchange channel (Staros, 1982*a, b*).

The high yield of band 3 dimers cross-linked at the extracytoplasmic face with BS^3 or with DTSSP has allowed us to extend our study of the topology of the extracytoplasmic domain of the anion exchange channel. We have combined our cross-linkers (Staros, 1982*b*) with chymotryptic cleavage of band 3 in intact erythrocytes (Steck et al., 1976) which results in cleavage of band 3 subunits into fragments of $M_r = 58{,}000$ and $M_r = 38{,}000$. Our results, together with the published data from several laboratories, are consistent with a head-to-head association of band 3 subunits, with the dimer having a twofold axis of symmetry perpendicular to the plane of the membrane.

Materials and Methods

Materials

Water for these experiments was deionized, then distilled in glass. BS^3 and DTSSP were synthesized as previously described (Staros, 1982*b*). Phenylmethylsulfonylfluoride and α-chymotrypsin, three times crystallized, were obtained from Sigma. All other reagents were ACS Certified grade or better.

Methods

Erythrocytes were prepared as prviously described (Staros & Richards, 1974) from fresh (≦1 week from drawing), volunteer-donated, human blood collected into heparin or into acid-citrate-dextrose. Cross-linking of erythrocytes with BS^3 or with DTSSP, preparation of membranes for electrophoresis, and $NaDodSO_4$-polyacrylamide gel electrophoresis in one dimension (Fairbanks et al., 1971) and in two dimensions (Wang & Richards, 1974) were carried out as previously described (Staros et al., 1981; Staros, 1982*b*). Chymotryptic digestion of intact erythrocytes was carried out as described by Steck et al. (1976), except that the enzyme concentration was 0.25 mg/ml and the digestion was terminated by addition of phenylmethylsulfonylfluoride (Fahrney & Gold, 1963) to 2 to 3 mM and incubation at room temperature for 1 hr. Oxidation of endogenous thiols by Cu^{2+}-*o*-phenanthroline in membranes prepared from chymotrypsin-treated cells was carried out as described by Wang and Richards (1974).

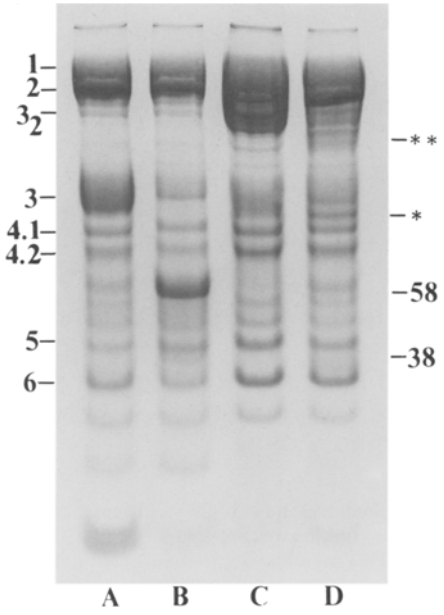

Fig. 1. Membranes from intact erythrocytes cross-linked with BS^3 and digested with chymotrypsin. Cells were treated as follows and then were lysed. The membranes isolated from those cells were disolved in solubilizing solution *with* DTT, and were subjected to electrophoresis in $NaDodSO_4$-polyacrylamide gels under *reducing* conditions. The photograph is of the Coomassie blue G-250 stained, wet gel. Lane *A*, no treatment; lane *B*, digested with chymotrypsin; lane *C*, cross-linked with 5 mM BS^3; lane *D*, cross-linked with 5 mM BS^3 then digested with chymotrypsin. Positions of bands 1, 2, 3, 4.1, 4.2, 5 and 6, the band 3 dimer (3_2), the $M_r = 58{,}000$ (58) and $M_r = 38{,}000$ (38) chymotryptic fragments, and products of sequential cross-linking and chymotryptic digestion (* and **) are marked. For details, *see* Materials and Methods

Results

When membranes from human erythrocytes are dissolved in an electrophoresis sample buffer containing $NaDodSO_4$ and DTT, subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis under reducing conditions, and stained with Coomassie blue G-250, the pattern obtained is as shown in Fig. 1, lane *A* (Fairbanks et al., 1971). When erythrocytes are treated with chymotrypsin overnight prior to the preparation of the membranes, the pattern obtained on $NaDodSO_4$-polyacrylamide gel electrophoresis is as shown in Fig. 1, lane *B* (Steck et al., 1976). The large majority of the Coomassie blue staining material which, in the control (lane *A*), migrates in the band 3, $M_r = 95{,}000$ region, has been removed by chymotrypsin treatment. A new band has appeared (lane *B*) at $M_r = 58{,}000$, which corresponds to the amino terminal fragment of the band 3 subunit (Steck et al., 1978). A very faint new band has also appeared at $M_r = 38{,}000$, corresponding to the

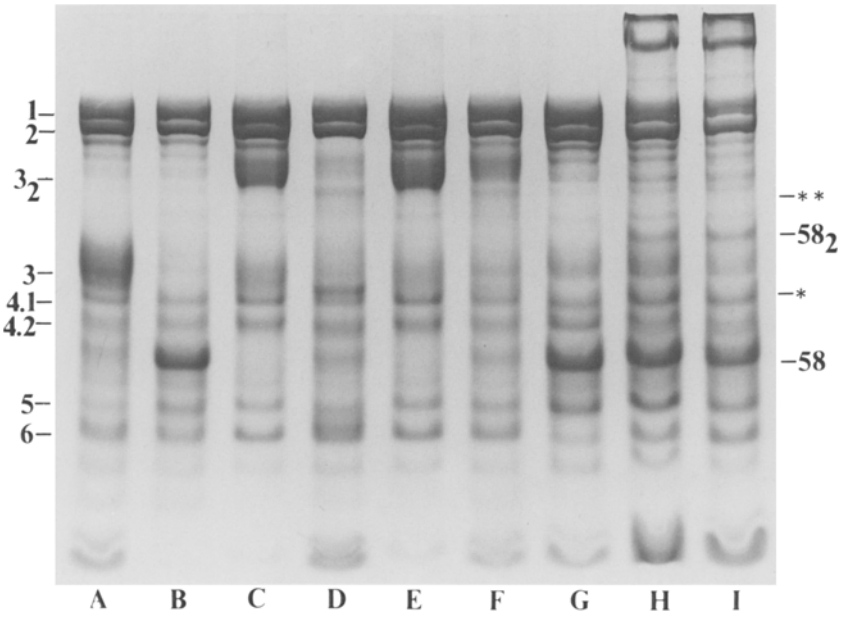

Fig. 2. Membranes from intact erythrocytes cross-linked with DTSSP and digested with chymotrypsin, compared with Cu^{2+}-*o*-phenanthroline-oxidized membranes from chymotrypsin-digested intact erythrocytes. Cells were treated as follows and then were lysed. The membranes isolated from these cells were dissolved in solubilizing solution *without* DTT, and were subjected to electrophoresis in $NaDodSO_4$-polyacrylamide gels under *nonreducing* conditions. One erythrocyte preparation was used for samples *A*–*F*, and a second preparation was used for samples *G*–*I*. The photograph is of the wet gel stained with Coomassie blue G-250. Lane *A*, no treatment; lane *B*, digested with chymotrypsin; lane *C*, cross-linked with 5 mM DTSSP; lane *D*, cross-linked with 5 mM DTSSP and subsequently treated with chymotrypsin; lane *E*, cross-linked with 10 mM DTSSP; lane *F*, cross-linked with 10 mM DTSSP and subsequently treated with chymotrypsin; lane *G*, treated with chymotrypsin as in lane *B*, but with the cell preparation used in lanes *H* and *I*; lanes *H* and *I*, cells digested with chymotrypsin, then treatment of the isolated membranes with Cu^{2+}-*o*-phenanthroline for 5 min (lane *H*) or 10 min (lane *I*). Positions marked include those in Fig. 1 and the dimer of the $M_r = 58{,}000$ fragment (58_2). For details, *see* Materials and Methods

carboxy terminal fragment of the band 3 subunit (Steck et al., 1978). When erythrocytes are treated with the noncleavable membrane-impermeant cross-linker BS^3, the pattern obtained is as shown in lane *C* (Staros, 1982*b*). The staining in the band 3 region is substantially reduced, and a new staining region at $M_r \sim 190{,}000$ has appeared, corresponding to the covalent dimer of band 3 subunits cross-linked at the extracytoplasmic membrane face (Staros, 1982*b*). When intact erythrocytes are first cross-linked with BS^3 and then treated with chymotrypsin prior to the preparation of membranes, the $NaDodSO_4$-polyacrylamide gel electrophoresis pattern obtained is that shown in Fig. 1, lane *D*. Much of the material in the band 3 region has been removed, and the fraction of chymotrypsin-cleaved band 3 migrating as free $M_r = 58{,}000$ amino terminal fragment is greatly reduced in comparison with that in Fig. 1, lane *B*. A new sharp band has appeared at $M_r = 90{,}000$ (labeled *), as has a region centered at $M_r \sim 140{,}000$ (labeled **). In addition, some material migrates in the region of band 3 dimer.

Similar results to those described above are obtained in experiments in which the membrane-impermeant, cleavable cross-linker DTSSP is substituted for the noncleavable reagent BS^3 and the membranes from treated cells are subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis under nonreducing conditions. In Fig. 2, the products of DTSSP cross-linking and chymotryptic digestion of band 3 in intact erythrocytes are compared with those from chymotrypsin treatment of intact cells followed by Cu^{2+}-*o*-phenanthroline catalyzed oxidation of the isolated membranes from such cells. Lanes *A*–*D* parallel the same lanes in Fig. 1, i.e., lane *A* is a control (no treatment), lane *B* is chymotrypsin treatment only which produces the $M_r = 58{,}000$ band, lane *C* is cross-linking with DTSSP (5 mM) only which produces a band corresponding to a dimer of band 3, and lane *D* is cross-linking with DTSSP (5 mM) followed by chymotrypsin treatment which produces a new band at $M_r = 90{,}000$, a region centered at $M_r \sim 140{,}000$, and staining in the region of the band 3 dimer. Increasing the concentration of DTSSP to 10 mM increases the relative yield of higher molecular weight products, either in the case of cross-linking

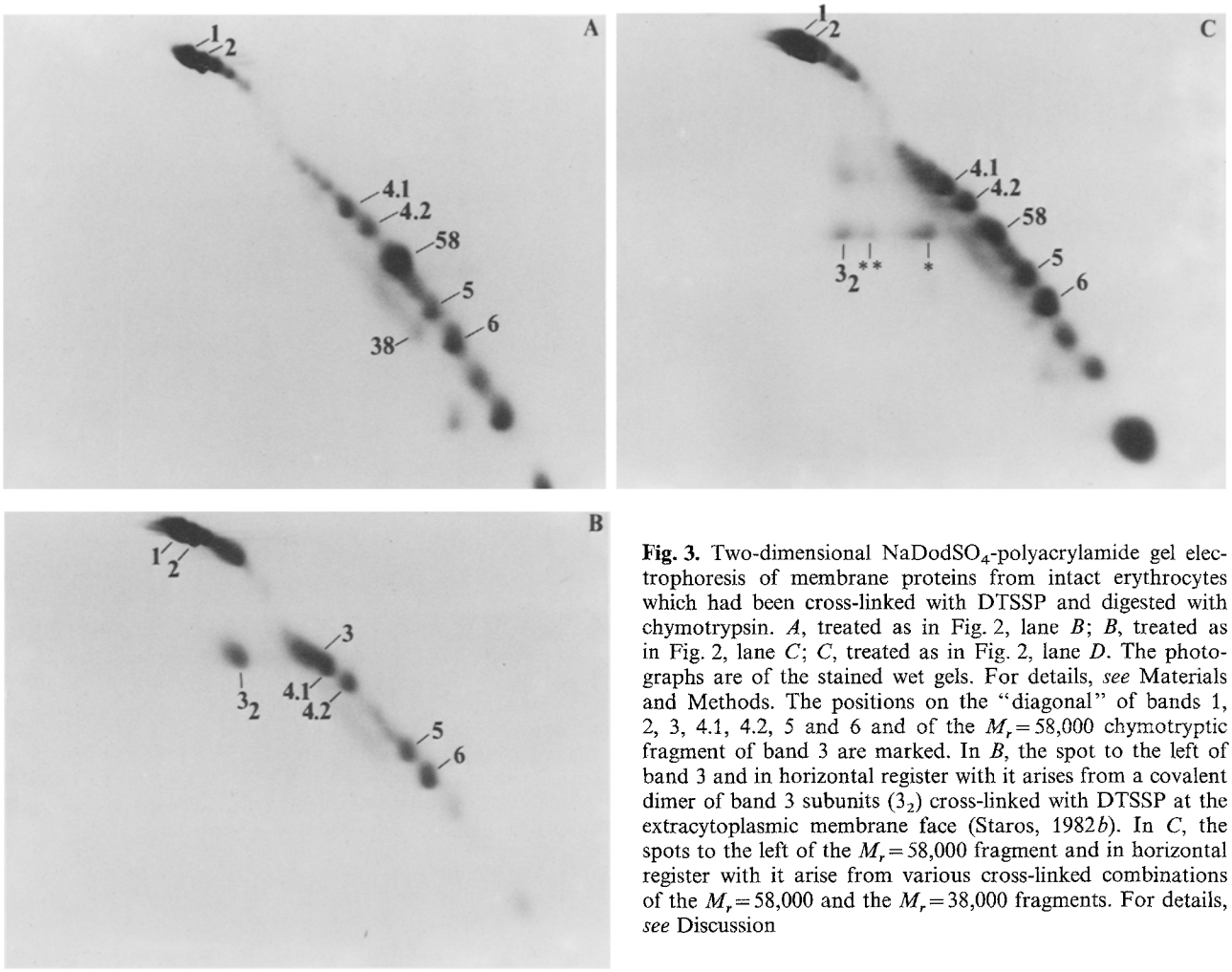

Fig. 3. Two-dimensional $NaDodSO_4$-polyacrylamide gel electrophoresis of membrane proteins from intact erythrocytes which had been cross-linked with DTSSP and digested with chymotrypsin. *A*, treated as in Fig. 2, lane *B*; *B*, treated as in Fig. 2, lane *C*; *C*, treated as in Fig. 2, lane *D*. The photographs are of the stained wet gels. For details, *see* Materials and Methods. The positions on the "diagonal" of bands 1, 2, 3, 4.1, 4.2, 5 and 6 and of the $M_r = 58{,}000$ chymotryptic fragment of band 3 are marked. In *B*, the spot to the left of band 3 and in horizontal register with it arises from a covalent dimer of band 3 subunits (3_2) cross-linked with DTSSP at the extracytoplasmic membrane face (Staros, 1982*b*). In *C*, the spots to the left of the $M_r = 58{,}000$ fragment and in horizontal register with it arise from various cross-linked combinations of the $M_r = 58{,}000$ and the $M_r = 38{,}000$ fragments. For details, *see* Discussion

alone (lane *E*) or cross-linking followed by chymotrypsin treatment (lane *F*). In particular, lane *F* has much more material than lane *D* running in the band 3 dimer region and much less running in the $M_r = 90{,}000$ band. The samples in lanes *A*–*F* were prepared from a single blood sample. The Cu^{2+}-*o*-phenanthroline cross-linking (lanes *G*–*I*) was carried out with cells from a second sample. The sample in lane *G* results from treatment of the cells with chymotrypsin under the same conditions as were used to prepare the sample in lane *B*. Treatment of membranes from chymotrypsin-treated cells by Cu^{2+}-*o*-phenanthroline for 5 min (lane *H*) or 10 min (lane *I*) results in covalent dimers of the $M_r = 58{,}000$ amino terminal fragment, linked by a disulfide bond formed between cysteinyl residues which are in the cytoplasmic domain of the band 3 subunits (Steck et al., 1976). None of the products of DTSSP cross-linking and chymotryptic digestion correspond to this dimer of $M_r = 58{,}000$ fragments.

In Fig. 3 are shown patterns from the two-dimensional $NaDodSO_4$-polyacrylamide gel electrophoresis of samples prepared as in Fig. 2, lanes *B* (intact cells treated with chymotrypsin), *C* (intact cells cross-linked with 5 mM DTSSP) and *D* (intact cells cross-linked with 5 mM DTSSP, then digested with chymotrypsin). In this electrophoretic system (Wang & Richards, 1974), samples are subjected to electrophoresis under nonreducing conditions in the first dimension (to maintain the disulfide bond in the cross-linker) and under reducing conditions in the second dimension (to cleave the disulfide bond in the cross-linker). In this system, proteins which had not been cross-linked fall on a "diagonal" in the final electropherogram. Products of cross-linking fall to the left of the "diagonal." In Fig. 3*C* it is shown that all three molecular weight classes of products of cross-linking and chymotrypsin cleavage, i.e., those at $M_r = 90{,}000$, at $M_r \sim 140{,}000$ and at $M_r \sim 190{,}000$, contain copies of the $M_r = 58{,}000$ amino terminal fragment of band 3.

Discussion

The results presented here indicate that cross-links formed by reaction of BS^3 or of DTSSP with the extracytoplasmic domain of the human erythrocyte anion exchange channel are formed between portions of the band 3 polypeptide corresponding to the $M_r = 58{,}000$ amino terminal chymotryptic fragment and the $M_r = 38{,}000$ carboxy terminal chymotryptic fragment. Such cross-linking between unlike portions of the band 3 polypeptide is consistent with the intrasubunit as well as the intersubunit cross-linking observed. Several lines of evidence support this conclusion.

In experiments in which intact cells were first cross-linked with BS^3 or with DTSSP (at 5 mM), then were digested with chymotrypsin (Fig. 1, lane *D*; Fig. 2, lane *D*, respectively), a prominent product migrates at $M_r = 90{,}000$. We interpret this product as resulting from the cross-linking of one $M_r = 58{,}000$ fragment with one $M_r = 38{,}000$ fragment in an arrangement which results in a $NaDodSO_4$-protein complex of somewhat smaller Stoke's radius than is formed by dissolution of intact band 3 in $NaDodSO_4$. The sharpness of the $M_r = 90{,}000$ band as compared with breadth of unmodified band 3 (lane *A* in Figs. 1 and 2) is somewhat puzzling. Heterogeneity in the carbohydrate attached to the portion of band 3 corresponding to the $M_r = 38{,}000$ fragment has been suggested as the cause for the diffuse appearance of band 3 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Yu & Steck, 1975; Drickamer, 1978). One might expect therefore, that the product of cross-linking one $M_r = 58{,}000$ fragment with one $M_r = 38{,}000$ fragment might be equally diffuse. One possibility is that the $M_r = 90{,}000$ products result from the cross-linking of $M_r = 58{,}000$ fragments with a particular subset of $M_r = 38{,}000$ fragments, with other products of $M_r = 58{,}000$ to $M_r = 38{,}000$ cross-linking migrating at higher M_r. That this could be the case is suggested by the somewhat greater amount of staining material in the band 3 region of lane *D* as compared with lane *B* in both Figs. 1 and 2 and the breadth of the band marked * in Fig. 3*C*. In any case, the sharpness of the $M_r = 90{,}000$ band in gels run under reducing conditions (Fig. 1*D*) suggests that this may be one discrete product, resulting from the cross-linking of a particular pair of lysyl residues, or at most a small number of products of nearly identical Stoke's radius in $NaDodSO_4$. We cannot, however, ascertain at this point whether the $M_r = 90{,}000$ band is the product of intersubunit or intrasubunit cross-linking, or both.

It is interesting to compare our results with those of Jennings and Passow (1979), who demonstrated that band 3 cleaved with chymotrypsin could be virtually quantitatively reconstituted to $M_r = 95{,}000$ by treatment with H_2DIDS, a bifunctional affinity inhibitor of anion transport, and incubation at pH 9.5. Treatment of intact erythrocytes not digested with chymotrypsin with H_2DIDS and high pH does not result in the production of band 3 dimers, suggesting that the interfragment cross-linking observed by these workers was between two fragments of the same subunit (Jennings & Passow, 1979).

A second set of products in these experiments migrates as a set of bands in a zone around $M_r \sim 140{,}000$. We interpret the products which migrate as bands in this zone as arising from the cross-linking of various combinations of three of the four chymotryptic fragments from one band 3 dimer, produced by the cross-linking of an additional fragment to the $M_r = 90{,}000$ complex. The range of M_r spanned by this set of bands suggests that both 58-38-58 and 38-58-38 products are formed. This interpretation is supported by a comparison of the products of DTSSP cross-linking and chymotryptic digestion of intact erythrocytes with those from the Cu^{2+}-*o*-phenanthroline catalyzed oxidation of membranes from chymotrypsin-digested erythrocytes. Oxidation of erythrocyte membranes catalyzed by Cu^{2+}-*o*-phenanthroline has been demonstrated to result in the formation of covalent dimers of band 3 subunits formed by oxidation of a pair of cysteinyl residues (Steck, 1972) in the cytoplasmic domains (Steck et al., 1976) of two band 3 subunits. Cu^{2+}-*o*-phenanthroline catalyzed oxidation of membranes prepared from chymotrypsin-digested erythrocytes results in the formation of a dimer of the $M_r = 58{,}000$ amino terminal fragments (Steck et al., 1976) (Fig. 2, lanes *H* and *I*). The sharp band corresponding to the dimer of $M_r = 58{,}000$ fragments does not comigrate with any significant product of DTSSP cross-linking and chymotryptic digestion. This supports our contention that the interfragment cross-links produced by DTSSP at the extracytoplasmic membrane face are between unlike fragments, because a homodimer of $M_r = 58{,}000$ fragments would be an expected intermediate (Peters & Richards, 1977) for any trimers or tetramers of fragments having 58-58 cross-links. A third set of products migrates in the position of the band 3 dimers. This corresponds to the cross-linking of all four chymotryptic fragments of the band 3 dimer.

That the $M_r = 58{,}000$ fragment is a part of all

of the cross-linked products discussed above, i.e., the band of $M_r = 90{,}000$ and the sets of bands at $M_r \sim 140{,}000$ and $M_r \sim 190{,}000$, is demonstrated by the two-dimensional electrophoretic separation shown in Fig. 3*C*. Spots in horizontal register with the $M_r = 58{,}000$ fragment are seen in vertical register with the positions on the diagonal corresponding to the three product classes. Also in vertical register with these spots are a set in horizontal register with the $M_r = 90{,}000$ position on the diagonal and (more faintly) others in register with the $M_r = 140{,}000$ position on the diagonal. These result from the incomplete reduction of disulfide bonds in DTSSP during electrophoresis in the second dimension, as demonstrated by the following experiment. A sample of membranes was prepared identically with those used for Fig. 3*C*, except that they were dissolved in the presence of DTT to cleave the cross-links. When this sample was subjected to two-dimensional $NaDodSO_4$-polyacrylamide gel electrophoresis, the pattern obtained was indistinguishable from that in Fig. 3*A* (*data not shown*), demonstrating that all of the off-diagonal spots in Fig. 3*C* arise from interfragment cross-linking and that the off-diagonal spots at $M_r > 58{,}000$ in Fig. 3*C* arise from incomplete reduction of disulfide bonds in the cross-links[2]. This control also demonstrates that cross-linking with DTSSP prior to chymotryptic digestion does not affect the production of chymotryptic fragments, at least to the limits of detection of our system.

Two important caveats must be borne in mind in evaluating our interpretation. First, our analysis is based on positive evidence for the $M_r = 58{,}000$ amino terminal chymotryptic fragment in the three size classes of cross-linked products. We are unable, using the present methods, to make the corresponding analysis of the presence of the $M_r =$ 38,000 carboxy terminal chymotryptic fragment, because like other groups (Cabantchik & Rothstein, 1974; Steck et al., 1976; Jennings & Passow, 1979), we find that the yield as estimated by coomassie blue stain of the $M_r = 38{,}000$ fragment is low and variable, though the actual ratio of $M_r =$ 38,000 fragment: $M_r = 58{,}000$ fragment has been shown to be 1:1 (Jennings & Passow, 1979). Second, as in all cross-linking experiments, only positive results are interpretable (Peters & Richards, 1977). Our experiments suggest that the $M_r =$ 58,000 fragment of one subunit of the anion exchange channel dimer is proximal to both the $M_r =$ 38,000 fragment of the same subunit and to the $M_r = 38{,}000$ fragment of the other subunit of the dimer. While we observe no cross-linking between $M_r = 58{,}000$ fragments of the two subunits of the anion exchange channel, we cannot interpret our data as indicating that these have no mutual contact. We can only observe that no detectable cross-links are formed between them under our conditions.

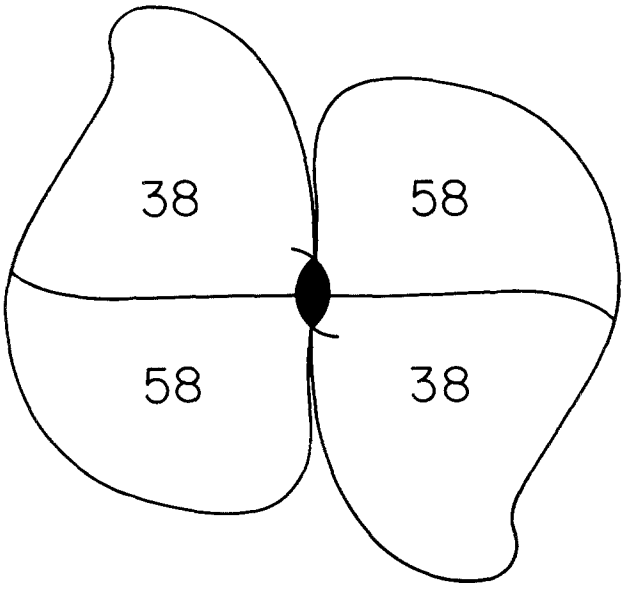

Fig. 4. Schematic diagram of the topology of the extracytoplasmic domain of the human erythrocyte anion exchange channel. The perspective is looking "down" onto the extracytoplasmic surface of the membrane. ◗, twofold (C_2) axis of symmetry

With these limitations in mind, we can make some useful interpretations of our data. Our data, which are consistent with the assumption that the anion exchange channel dimer has an axis of symmetry orthogonal to the membrane (Klingenberg, 1981), are schematically summarized in Fig. 4. While the shapes chosen for the fragments are arbitrary, the extent of contact between areas representing the chymotryptic fragments of band 3 is meant to symbolize the extent of cross-linking that we observe between those fragments. Thus, our data are consistent with a head-to-head association of band 3 subunits, such that the anion exchange channel has a twofold axis of symmetry perpendicular to the plane of the membrane.

In some cross-linking studies of erythrocyte membrane proteins, notably those in which isolated erythrocyte membranes are treated with Cu^{2+}-*o*-phenanthroline to catalyze the formation of disulfide bonds from endogenous thiols, oli-

[2] We have observed this phenomenon in cross-linking experiments with other proteins. In general, when an oligomeric protein is highly cross-linked with DTSSP, it is very difficult to cleave all of the cross-links by electrophoresis in this two-dimensional system.

gomers of band 3 subunits up to tetramers have been observed, suggesting that the anion exchange channel might be tetrameric in situ (Wang & Richards, 1974; Palek, Liu & Liu, 1978). Such studies are of necessity carried out with membranes rather than with intact erythrocytes, and therefore the tetramers formed might be an artifact of disruption of the membrane. However, recent preliminary evidence obtained by quantitative electron microscopy independently supports the tetrameric model for band 3 in situ (Weinstein, Khodadad & Steck, 1980).

The data presented here, as well as previous data from this laboratory on the proximity of the extracytoplasmic domains of band 3 subunits (Staros et al., 1981; Staros, 1982*b*) do not directly address the issue of whether the anion exchange channel is composed of two or four band 3 subunits. Our data do, however, place certain limits on the tetramer model. Our data cannot be reconciled with a model in which four subunits are related by a fourfold axis of symmetry perpendicular to the membrane plane. If the tetramer exists, our data suggest that this structure would be composed of two of the dimeric units schematically represented in Fig. 4. If these two dimeric units are symmetrically related, the twofold axis of symmetry would have to be a different one from that which relates the two subunits of the dimer.

A possible model which might reconcile the dimer and tetramer models would be one in which the extracytoplasmic and transmembrane domains exist as dimers and the cytoplasmic domains of the dimers, corresponding to the amino terminal $M_r = 41{,}000$ tryptic fragments of the band 3 subunits (Steck et al., 1976, 1978), associate to form a dimer-of-dimers, $(\alpha_2)_2$, structure.

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