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Modification by Papain of the Structure and Function of Band 3, the Erythrocyte Anion Transport Protein†

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ABSTRACT: Extracellular papain is known to inhibit the anion transport function of the band 3 protein of the human red blood cell membrane. Previous work [Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 554*, 498–519] had suggested that this inhibition may result from the removal by papain of 5000–10 000 daltons from the 35 000-dalton chymotryptic peptide of band 3. The present work shows, however, that papain also removes a small peptide from the C terminus of the 60 000-dalton chymotryptic peptide. The C-terminal amino acid sequence of this peptide is -Lys-Thr-Tyr. Whether or not this newly discovered action of papain is responsible for inhibiting anion transport is unknown. The effects of extracellular papain on the band 3 function have been characterized in detail. Papain inhibits Cl–Cl exchange in a high Cl medium by almost 90%. This inhibition appears to result from inhibition of the efflux step in the catalytic cycle for the transport, because papain does not inhibit the anion transport when it is assayed under influx-limited conditions. Moreover, since papain has no detectable effect on the dissociation constant for extracellular substrate (SO_4) binding, the material removed by papain cannot be involved closely in the outward-facing substrate site. In contrast, removal of this material strongly (12-fold) reduces the affinity of the inhibitor 4,4′-dinitro-2,2′-stilbenedisulfonate for outward-facing sites. Therefore, stilbenedisulfonate binding involves portions of the band 3 molecule which are not intimately related to substrate binding.

Band 3 (Fairbanks et al., 1971) is the major integral membrane protein of the human red blood cell. It has a subunit molecular weight of about 95 000 and functions in the catalysis of anion transport (Cabantchik & Rothstein, 1974a; Passow et al., 1975; Ho & Guidotti, 1975), the physiological mode of which is Cl–HCO_3 exchange. Despite recent advances in the understanding of both the structure and function of band 3 [see Steck (1978) and Knauf (1979)], very few relationships between structure and function have been established.

Analysis of the products of in situ enzymatic proteolysis of band 3 is a potential source of structure–function information. The advantages of in situ proteolysis are that the sidedness of the action of the enzyme on band 3 can be controlled, proteolysis is limited to a small number of sites, and the transport function of the protein can be assayed after the proteolysis. Since band 3 is a major component of the membrane, large cleavage products are relatively easy to recover and isolate.

It is well established that extracellular chymotrypsin cleaves band 3 into two peptides, both of which remain firmly attached to the membrane (Cabantchik & Rothstein, 1974b; Steck et al., 1978; Markowitz & Marchesi, 1981). These peptides have approximate molecular weights of 60 000 and 35 000, and they very likely remain associated with each other in a native complex after the proteolysis (Jennings & Passow, 1979; Reithmeier, 1979). Chymotrypsin treatment does not affect the transport function of the protein. In contrast, extracellular papain strongly inhibits both Cl–Cl and SO_4–SO_4 exchange (Passow et al., 1977; Ku et al., 1979). Jennings & Passow (1979) showed that this inhibition may be related to the degradation by papain of the 35 000-dalton chymotryptic peptide, although removal of material (less than or equal to six residues) from the 60 000-dalton peptide could not be ruled out.

The present results show that papain does in fact remove a small peptide from the 60 000-dalton peptide and that the elementary kinetic step which is inhibited by papain is anion efflux. We also demonstrate that papain does not alter substrate (SO_4) binding affinity at the extracellular surface but that it does strongly reduce the affinity for the disulfonic stilbene inhibitor 4,4′-dinitro-2,2′-stilbenedisulfonate (DNDS).[1]

Materials and Methods

Chemicals. Sources of reagents were the following: enzymes from Boehringer Mannheim, Indianapolis, IN; PMSF (phenylmethanesulfonyl fluoride), Sephadex G10, and Sepharose 6B from Sigma, St. Louis MO; $^{35}SO_4$ from New England Nuclear, Boston, MA; ^{36}Cl from ICN Radiochemicals, Irvine, CA; DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonate) from Pierce, Rockford, IL. The dihydro analogue of DIDS (H_2DIDS) was a generous gift of Professor H. Passow. DNDS (technical; Aldrich, Milwaukee, WI) was purified by adsorption to Sephadex G10 in aqueous 100 mM Na_2SO_4, followed by desorption with distilled water.

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[1] Abbreviations used: DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonate; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; CPA, carboxypeptidase A; Na-DodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid.

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Enzyme Treatment of Red Cells. Adult human red blood cells were obtained from the Lipid Research Lab of the University of Iowa; cells were used after at most 4 days of storage at 4 °C (EDTA anticoagulant). Cell preparations for ion flux measurements were identical with those in which the band 3 peptides were isolated. Cells were washed and incubated with 1 mg/mL bovine pancreatic chymotrypsin for 1 h at 37 °C and pH 7–7.4 as described earlier (Jennings & Passow, 1979). Papain digestion of the chymotrypsin-treated cells was at pH 7, 37 °C, 1 mg/mL papain, in 150 mM KCl, 10 mM sodium phosphate, and 2 mM cysteine. Following the enzyme treatment(s), cells were washed twice in phosphate-buffered 150 mM KCl, pH 7, containing 0.1% BSA and 30 μg/mL PMSF, and then washed twice in the same buffer without PMSF.

Ion Flux Measurements. The band 3 catalyzed anion exchange fluxes in cells prepared as described above were assayed in four ways. In all four assays, the initial intracellular Cl concentration was about 150 mM and the initial intracellular pH about 7.0. The rate constant for Cl–Cl equilibrium exchange in 150 mM KCl–10 mM sodium phosphate, pH 7, was measured at 0 °C by the inhibitor (30 μM H_2DIDS or DIDS) stop method described previously (Ku et al., 1979). The same method was used to measure net Cl loss at 27 °C into Cl-free, HCO_3-free (30 min N_2 bubbled) 140 mM sodium phosphate, pH 6. In the Cl–Cl exchange measurements, samples were centrifuged after no more than 1 min in the stop solution. Cellular Cl contents (millimoles per kilogram of cell solids) were determined as follows. One volume of buffered KCl containing a known amount of ^{36}Cl (usually 1 μCi/mL) was added to 2 volumes of packed cells and incubated a few minutes at 0 °C to allow ^{36}Cl equilibration between cells and medium. Cellular Cl content was calculated from the extracellular Cl concentration, hematocrit, cellular dry weight, and the initial and final extracellular radioactivity. The enzyme treatments did not significantly alter the cellular Cl content. Net SO_4 influx (Cl–SO_4 exchange) was measured by the cold stop method described previously (Jennings, 1980), except that 0.1% BSA was included in the stop solution.

Chloride–chloride exchange at low (0.6 mM) extracellular Cl concentration was measured as tracer influx rather than efflux. Packed cells (0.2 mL) were added to 15 mL of ice-cold 200 mM sucrose, 20 mM sodium citrate, pH 6, and 0.5 mM Cl containing 0.05 μCi of ^{36}Cl. Aliquots were removed at timed intervals with a repeating syringe and injected into 0.5-mL stop solution as for the efflux measurements. The samples were spun 1 min in an Eppendorf microfuge, and the supernatant was aspirated through a 22-gauge needle, without removal of any cells. The ^{36}Cl radioactivity in the pellets was determined after lysis in 0.7 mL of water and precipitation of hemoglobin with 0.5 mL of 21% Cl_3CCOOH. It was not necessary to wash the pellet free of extracellular ^{36}Cl, since the intracellular Cl concentration was 250 times the extracellular concentration. Control experiments demonstrated that net Cl loss during the Cl–Cl exchange measurements did not exceed 2%.

Isolation of the 60 000-Dalton Peptide. Following the enzyme treatments, cells were washed twice in 150 mM $NaHCO_3$, 0.1% BSA, and 30 μg/mL PMSF and then twice in 150 mM $NaHCO_3$. Cells were then lysed in 20 volumes of 5 mM $NaHCO_3$ (pH about 8.2) at 0–0.5 °C and washed in the same (cold) medium until white. Peripheral membrane protein was removed by stripping with 0.1 N NaOH at 0 °C (Steck & Yu, 1973) followed by two washes in 5 mM $NaHCO_3$. Membranes were solubilized by heating 3 min at 100 °C in an equal volume of 4% $NaDodSO_4$–80 mM dithiothreitol. The 60 000-dalton band 3 peptide was isolated by molecular exclusion chromatography on a Sepharose 6B column (1.6 × 38 cm) in 20 mM NaCl, 5 mM $NaHCO_3$, and 0.1% $NaDodSO_4$, after Steck et al., (1978). Fractions containing pure 60 000-dalton peptide were identified by polyacrylamide gel electrophoresis in $NaDodSO_4$ (Jennings & Passow, 1979). A typical preparation yielded 3 mg of purified 60 000-dalton peptide from 15 mL of red cells.

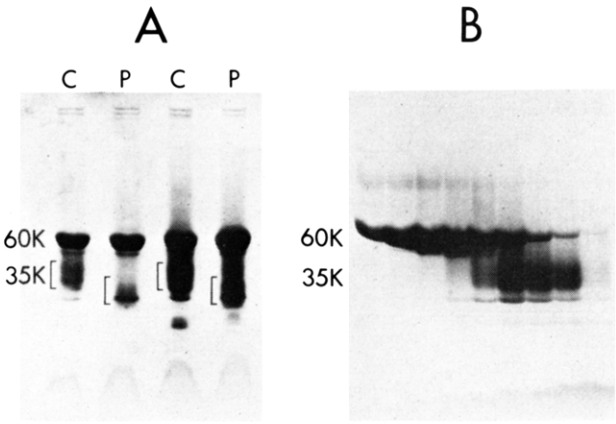

FIGURE 1: (A) Polyacrylamide slab gel (4–30% acrylamide, 0.1% $NaDodSO_4$) of protein in NaOH-stripped red blood cell membranes from cells treated with chymotrypsin (C) or with chymotrypsin and then papain (P). The two lanes on the left contain about 25 μg of protein; the other two lanes contain 50 μg of protein. The positions of the 60 000-dalton (60K) and 35 000-dalton (35K) chymotryptic peptides of band 3 are indicated. The 35K band covers a broad range of molecular weights (35 000–45 000), very likely because of its heterogeneous carbohydrate moiety [see Markowitz & Marchesi (1981)]. (B) Polyacrylamide gel electrophoretogram of 50-μL aliquots from successive fractions eluted from Sepharose 6B as described under Materials and Methods. The sample applied to the colum consisted of reduced, solubilized (after NaOH stripping) membranes from chymotrypsin-treated red cells. The fractions shown are (from left) fractions 21–29 for a column in which the void volume was at fraction 13.

Fractions of interest were pooled and concentrated to a volume of 1.5 mL by ultrafiltration (Amicon YM5). Carboxypeptidase A digestion was carried out at 25 °C in 0.2 M $NaHCO_3$–0.1% $NaDodSO_4$, with repeat additions of 20 μg of enzyme to 3 mg of peptide at 1-h intervals. The carboxypeptidase A crystals were washed twice in distilled water before dissolution in the digestion buffer. Free amino acids were separated from the digestion mixture by Amicon YM5 ultrafiltration. Amino acid analysis was performed on a Beckman 121M amino acid analyzer, using norleucine as an internal standard in all samples. Results were expressed as moles of amino acid per mole of peptide. Protein was assayed by the Peterson (1977) modification of the method of Lowry et al. (1951).

Results

Papain Removes Material from the 60 000-Dalton Chymotryptic Peptide. Figure 1A shows the peptides of band 3 produced by extracellular chymotrypsin alone, or chymotrypsin followed by papain. As found earlier under slightly different conditions (Jennings & Passow, 1979), papain removes material from the 35 000-dalton peptide but does not detectably alter the electrophoretic mobility of the 60 000-dalton peptide. The orientation of the 60 000-dalton fragment is such that its C terminus is exposed on the outer surface of the membrane, and the N terminus is on the inner surface (Steck et al., 1976, 1978). Therefore, if extracellular papain has any effect on this peptide, the C-terminal amino acid(s) should be altered.

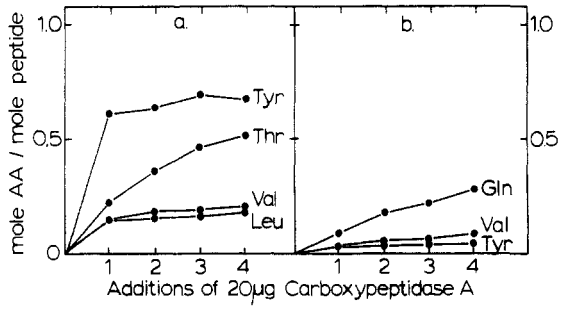

FIGURE 2: Amino acids released by successive additions of 20 μg of carboxypeptidase A at 1-h intervals to 3 mg of the purified 60 000-dalton band 3 peptide from red cells treated with (a) chymotrypsin alone or (b) chymotrypsin followed by papain.

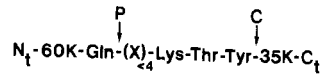

FIGURE 3: Partial sequence of the exofacial region of band 3 near the sites of chymotrypsin and papain cleavage.

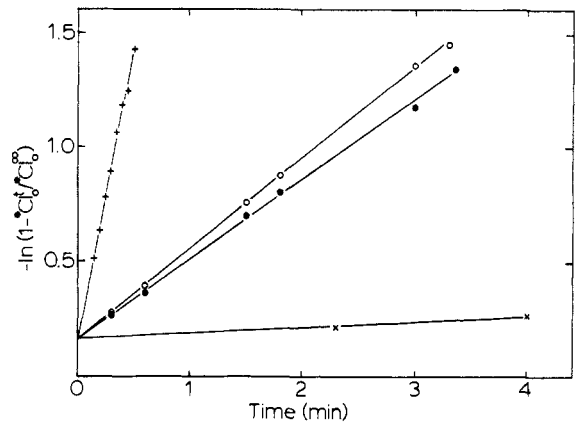

FIGURE 4: Semilogarithmic plot of ^{36}Cl efflux from red cells treated with chymotrypsin only (+), chymotrypsin followed by a single papain treatment (○), or chymotrypsin followed by two successive papain treatments (●). The lower points (×) refer to the same cells as ●, except the ^{36}Cl efflux was into a 140 mM sodium phosphate medium rather than the 150 mM Cl medium.

The 60 000-dalton peptide from cells treated with chymotrypsin alone was isolated (Figure 1B) and digested with carboxypeptidase A in 0.1% $NaDodSO_4$. We found that CPA loses activity rapidly in the detergent; this is undoubtedly why Steck et al. (1978) found only tyrosine released from the C terminus of the chymotryptic 60 000-dalton peptide after a 24-h incubation with CPA in $NaDodSO_4$. The denaturation problem was overcome by repeat additions of enzyme at 1-h intervals, keeping the peptide/enzyme molar ratio above 10.

In agreement with others (Drickamer, 1976; Steck et al., 1978), we find that tyrosine was released rapidly from the chymotryptic peptide (Figure 2a). Subsequent enzyme additions showed further release of threonine, also reported by Drickamer (1976). The yield of threonine was sufficient to indicate that it is the penultimate residue. Other than tyrosine and threonine, only leucine and valine were released in significant quantities, but the pattern of release suggests that these may result from an impurity. After four CPA additions, a single addition of carboxypeptidase B was made; the only amino acid released after 30 min was lysine, with a yield of 0.5 mol/mol of peptide, i.e., comparable to that of threonine. We therefore assign lysine as the residue beyond threonine.

The pattern of amino acid release from the 60 000-dalton peptide isolated from cells treated with chymotrypsin and then papain is completely different (Figure 2b). Only glutamine is released in significant quantity. These results show that papain *does* remove a small peptide from the 60 000-dalton chymotryptic peptide.[2] Previous work indicated that this small peptide could consist of at most about six residues (Jennings & Passow, 1979). This information, combined with the present data, allows the construction of a partial map of an exofacial segment of the band 3 protein (Figure 3). The only known residues in this segment are hydrophilic; this is not unexpected, since this material is in contact with extracellular water. We made no attempt to recover this peptide; it is probably released from the membrane and perhaps further degraded by papain.

Papain Does Not Completely Inhibit Band 3 Function. It is of interest to try to specify which elementary kinetic step in the anion transport is inhibited by papain. Possibilities include the destruction of the substrate binding site or prevention of the inward or outward translocation step. If papain completely inhibited band 3 function, it would be impossible to determine what elementary step is affected, because no assay of function could be made on the treated protein. Fortunately, papain does not completely inhibit the transport (Cl–Cl exchange at 0 °C), as shown in Figure 4. A single 1-h exposure of 1 mg/mL papain (after chymotrypsin) inhibits the Cl transport 86%. A subsequent identical treatment with fresh enzyme brings the inhibition to 88%. The residual Cl flux after two papain treatments does not represent a nonspecific leak caused by papain, because replacement of extracellular Cl by phosphate, a slowly transported anion, further reduces the ^{36}Cl efflux by a factor of 20. We conclude that the 1-h papain treatment cleaves all copies of band 3 at the site(s) responsible for inhibition and that the cleaved protein carries out Cl–Cl exchange at 12% of the original rate. (The possibility that 12% of the band 3 is not susceptible to papain is unlikely for reasons outlined under Discussion.) It is therefore possible in principle to determine which elementary kinetic event is inhibited.

Measurement of Band 3 Function under Influx-Limited Conditions. The anion transport catalyzed by band 3 is an obligatory one for one exchange [see Knauf (1979)]; band 3 may therefore be considered kinetically to be a two-substrate enzyme, the substrates being an intracellular and an extracellular anion. There is now considerable evidence that the catalytic cycle for the anion exchange is that of a ping-pong mechanism, in which an initially intracellular anion is transported outward and dissociates from the membrane, *and then* an extracellular anion binds and is transported inward (Gunn & Fröhlich, 1979; Jennings, 1980; Knauf, 1979). In the following, we assume that the ping-pong model is correct and that binding is much faster than translocation. The unimolecular rate constants for the outward Cl translocation event and its reverse, the inward Cl translocation, are not known and need not be equal. Accordingly, the inhibition by papain of Cl–Cl exchange (Figure 4) could result from reduction in the rates of either the influx or the efflux step (or both). The Cl–Cl exchange experiment thus does not show which elementary step is inhibited.

It is possible, however, to assay band 3 function under conditions in which *influx* is the rate-limiting step. The band 3 catalyzed net exchange of intracellular Cl for extracellular phosphate or SO_4 is limited by the phosphate or SO_4 influx rather than by the Cl efflux (Jennings, 1980), because phosphate and SO_4 penetrate the membrane 10^{-3}–10^{-4} times as rapidly as Cl (Gruber & Deuticke, 1973; Schnell et al.,

[2] H. Passow (personal communication) has also found that extracellular papain alters the C terminus of the 60K peptide.

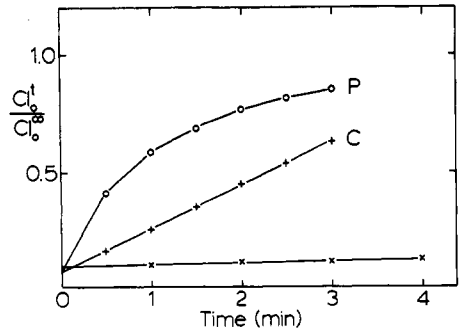

FIGURE 5: Time course of net Cl loss at 27 °C into 140 mM sodium phosphate, pH 6, from cells treated with chymotrypsin alone (+) or chymotrypsin followed by papain (○). The lower points (×) show the Cl loss from the same cells as ○, except that the medium contained 20 μM DIDS.

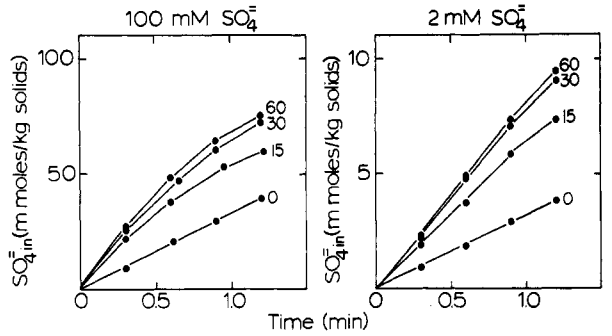

FIGURE 6: Time course of net SO_4 influx at 27 °C into Cl-loaded red cells resuspended in 100 mM Na_2SO_4–20 mM sodium citrate, pH 6.4 (left), or 2 mM Na_2SO_4, 20 mM sodium citrate, pH 6.4, and 200 mM sucrose (right). All cells were pretreated with chymotrypsin, and then with 1 mg/mL papain at 37 °C for 0, 15, 30, or 60 min as indicated.

1977). Figure 5 shows that papain does not inhibit net Cl–P_i exchange. The same papain treatment which inhibits Cl–Cl exchange by 86% actually accelerates net Cl–P_i exchange. The accelerated flux is still inhibited by at least 98% by 20 μM DIDS. Therefore, the transport step inhibited by the papain is *not* anion influx, since the transport is not inhibited when measured under influx-limited conditions.

To study band 3 function in papain-treated cells quantitatively under influx-limited conditions, we measured the net exchange of intracellular Cl for extracellular SO_4. The Cl–SO_4 exchange in red cells is an electroneutral exchange of one Cl for one SO_4 plus one proton (Jennings, 1976). Figure 6 shows the time course of net SO_4 influx into cells treated first with chymotrypsin (which has no significant effect on the net anion exchange) and then with 1 mg/mL papain for 0, 15, 30, and 60 min. Papain treatment accelerates the initial SO_4 influx by 2–3-fold in both low (2 mM) SO_4 and high (100 mM) SO_4 media. As for phosphate, the accelerated SO_4 influx is DIDS inhibitable (not shown). The SO_4 influx under these conditions is half-maximal at 10–20 mM extracellular SO_4 (M. L. Jennings, unpublished experiments); therefore, the acceleration at 100 mM SO_4 is clearly an effect on the inward translocation rate. The similar acceleration at 2 mM SO_4 suggests that papain has no effect on SO_4 binding to outward-facing transport sites on band 3.

Stilbenedisulfonate Binding Site. The residual Cl–Cl exchange flux as well as the acclerated Cl–P_i and Cl–SO_4 exchange fluxes in papain-treated cells is almost completely inhibited by high concentrations (20 μM) of DIDS. This shows that the stilbenedisulfonate binding site is not completely destroyed by the papain. To determine whether or not stilbenedisulfonate binding affinity is altered, we used DNDS,

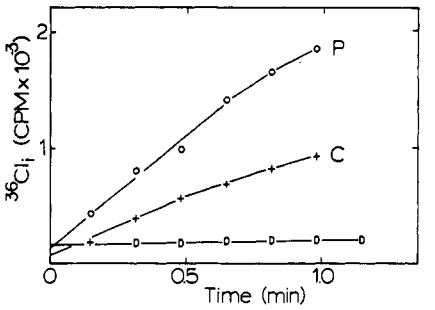

FIGURE 7: Tracer Cl influx at 0 °C from a 0.6 mM NaCl, 20 mM sodium citrate, and 200 mM sucrose medium into high Cl concentration (150 mM) red cells pretreated with chymotrypsin alone (+) or chymotrypsin followed by papain (○). The points (□) refer to the same cells as ○, except that the flux medium contained 20 μM DIDS.

which inhibits anion transport reversibly. The inhibitory effects of DNDS were measured on the net SO_4 influx from a low SO_4 (2 mM), very low Cl (<0.1 mM) medium into Cl-loaded cells. These assay conditions were chosen to maximize inhibitory potency: the outward 1000-fold Cl gradient should recruit virtually all of the transport systems into an outward-facing state (Knauf, 1979; Jennings, 1980), and the low SO_4 concentration minimizes the effects of competition.

The K_I for DNDS inhibition of the flux into chymotrypsin-treated cells is 0.1 μM; papain increases the K_I by a factor of 12 (not shown). Thus, the DNDS binding site is seriously altered by papain, even though the K_m for SO_4 binding to outward-facing transport sites is apparently not changed.

Cl–Cl Exchange at Low Extracellular Cl. The acceleration by papain of Cl–P_i and Cl–SO_4 exchange indicates that the elementary kinetic event inhibited by papain is outward translocation. The inhibition by papain of outward Cl translocation does not slow down net Cl–P_i or Cl–SO_4 exchange because, even if 10-fold slower than normal, the outward Cl translocation step is still much more rapid than inward P_i or SO_4 transport. If indeed the inhibited step is outward translocation, then papain should not inhibit Cl–Cl exchange in the presence of a large outward Cl gradient, because under these conditions the catalytic cycle should be limited mainly by extracellular Cl binding and inward translocation. Figure 7 shows the effect of papain on Cl–Cl exchange in a low (0.6 mM) Cl medium. In contrast to Figure 4, in which there is 86% inhibition in a high Cl medium, the Cl–Cl exchange flux in a low Cl medium is moderately accelerated (and still DIDS inhibitable). It appears, then, that the Cl influx step is accelerated, as are the P_i and SO_4 influx steps. Since papain acclerates Cl–Cl exchange by a factor of two at low extracellular Cl, but inhibits Cl–Cl exchange by 85% in the same cells (150 mM intracellular Cl) in a high Cl medium, it is obvious that the *apparent* affinity of band 3 for extracellular Cl is dramatically (15–20-fold) increased by papain. However, the change in apparent affinity does not imply a change in the *true* dissociation constant for extracellular Cl (see below).

Discussion

This paper represents the first attempt to correlate an irreversible structural modification of band 3 with changes in a particular elementary kinetic step in the anion transport. Using transport assays in which the influx steps of the transport cycle are rate limiting, we have shown the following:

(1) Papain digestion at the extracellular surface of band 3 does not inhibit the influx of SO_4, P_i, or Cl under digestion conditions which strongly inhibit Cl–Cl exchange in a 150 mM Cl medium. Therefore, the outward translocation step is very

likely the elementary step inhibited by the papain.

(2) This lack of inhibition of influx implies that the portions of band 3 which are removed by papain are *not* necessary for extracellular anion binding and inward translocation. These segments of band 3 are a small (<1000-dalton) hydrophilic peptide from the C terminus of the 60000-dalton chymotryptic peptide and a larger (5000–10000-dalton) portion of the 35000-dalton chymotryptic peptide.

(3) Papain does not cause a major change in the K_m for extracellular SO_4, but it reduces the inhibitory potency of DNDS by a factor of 12. This means that, although transported anions and disulfonic stilbenes compete for exofacial sites (Shami et al., 1978), DNDS binding must involve parts of the protein which are not intimately involved in substrate binding. This is not unexpected, since DNDS is physically much larger than substrate anions.

It is important to emphasize that we believe the papain-induced increase in the *apparent* affinity of band 3 for extracellular Cl is a consequence not of a real affinity change but of the reduced outward translocation rate constant and the accelerated inward translocation rate constant. From the kinetics of the ping-pong model (Gunn & Fröhlich, 1979; Knauf, 1979), it may be shown that the Cl–Cl exchange flux at a fixed intracellular Cl concentration will be half-maximal at an extracellular Cl concentration which is a function of the true Cl affinity as well as the inward and outward translocation rate constants. It is thus impossible in a single set of Cl–Cl exchange experiments to estimate the affinity for extracellular Cl, because the translocation rate constants are not known. For extracellular SO_4, however, estimates of the dissociation constant are possible because the measured Cl–SO_4 exchange flux at low extracellular Cl concentration is limited by SO_4 influx at all extracellular SO_4 concentrations (Jennings, 1980). Figure 6 shows that papain has little effect on the apparent binding constant for extracellular SO_4. Therefore, for at least one substrate anion, papain does not strongly alter binding at an outward-facing substrate site. Accordingly, we have no reason to suspect that papain causes a real change in Cl affinity for outward-facing sites. It is worth mentioning that if papain accelerates the inward translocation rate constant for Cl by 2-fold and inhibits the outward translocation rate constant by 10-fold (Figure 4), then the ping-pong model predicts that the apparent Cl affinity for outward-facing sites should be increased at least 10-fold, as is observed.

Previous work (Jennings & Passow, 1979) had strongly suggested that papain digestion of the 35000-dalton chymotryptic peptide of band 3 was responsible for transport inhibition. The present results show that this is not necessarily so, since papain also removes a small piece from the 60000-dalton peptide. We cannot say at present which action of papain is inhibitory to anion transport. Moreover, we do not know if the same cleavage site causes inhibition of efflux, acceleration of influx, and reduction of DNDS affinity. Extensive kinetic studies under more moderate digestion conditions will be necessary to answer these questions.

The ping-pong kinetic model of anion exchange was assumed to be the correct mechanism in the design and interpretation of these experiments. It should be noted, however, that our results provide further support for this mechanism. Papain treatment inhibits efflux and accelerates influx. The ping-pong model can explain these effects quite simply. The influx step is the unimolecular transition from an outward-facing to an inward-facing conformation of the protein–anion complex. If papain lowers the free energy of the inward-facing state and raises that of the outward-facing state (relative to that of the transition state), then the outward translocation rate is inhibited, and the inward translocation rate is accelerated. These are not, however, the only effects papain may have on the transporter. Our data do not exclude possible effects of papain on H^+ binding or on Cl or SO_4 binding to the inhibitory ("modifier") site [see Knauf (1979)].

Figure 4 shows that a second papain treatment has little effect on Cl–Cl exchange beyond the 86% inhibition of a single treatment. We believe that this indicates that the single treatment is sufficient to cleave virtually 100% of the copies of band 3 at the inhibitory site(s) and that the digested protein carries out Cl–Cl exchange (in a high Cl medium) at about 12% of the control rate. Another possible interpretation is that, for some reason, 12% of the band 3 is not susceptible to papain digestion. We reject this interpretation because of the following two findings. First, the papain-digested band 3 has a lower affinity for DNDS, but a Dixon plot of the inhibition is still linear. This would not be true if 12% of the band 3 still had a high affinity for DNDS. Second, a 1-h papain treatment increases the potency of extracellular Cl as an inhibitor of net SO_4 influx (Cl–SO_4 exchange) by about 15-fold (K_I = 0.2 mM vs. 3 mM for chymotrypsin-treated cells; data not shown). This is to be expected from the papain-induced increase in apparent affinity for extracellular Cl (Figure 7). Dixon plots (not shown) for Cl inhibition of the net SO_4 influx are linear for the cells treated either with chymotrypsin alone or with chymotrypsin and then papain. This is true even at high (95%) levels of inhibition in the papain-treated cells. If 12% of the band 3 had not been affected by papain, the Dixon plot should curve toward a much lower slope at high levels of inhibition. There is thus no evidence for a subset of the band 3 which is not susceptible to papain.

Acknowledgments

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Motions and Interactions of Phospholipid Head Groups at the Membrane Surface. 1. Simple Alkyl Head Groups†

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ABSTRACT: As a reference point for comparison with more complex head groups, a set of phospholipids with *simple* alkyl head groups has been studied. These analogues resemble the naturally occurring phospholipids, except they have phosphomethanol, -ethanol, -1-propanol, and -1-butanol as head groups. The gel-to-liquid-crystalline phase transition temperatures were measured with differential scanning calorimetry, and the phase properties of multilamellar dispersions were examined with phosphorus-31 NMR. The effect of the head-group size was found to be rather small. These lipids were synthesized with deuterium labels incorporated into the alcohol portion at all positions in the head groups except butanol, which was labeled only in the C-1 position. Determination of the ^2H residual quadrupole splittings led to an analysis of the head-group ordering properties. Specifically, these data showed that increasing the length of the head group leads to a more perpendicular orientation of the head group relative to the bilayer surface. Phosphorus-31 chemical shift anisotropy data were also compatible with this result. Measurement of surface pressure–area diagrams of monolayers of these compounds revealed that at high pressures (30 dyn/cm) all four lipids occupied similar areas (40–44 Å^2/molecule), yet at lower pressures, the larger the head group, the larger the occupied surface area. This result suggests that in a bilayer the fatty acyl chains occupy similar areas independent of the alkyl head-group size, and the larger head groups cannot pack properly without some conformational adjustment. The addition of phosphatidylcholine with its relatively bulky head group decreases the area available to the alkyl head groups, pushing the alkyl head groups out of the plane of the bilayer surface. Cholesterol, on the other hand, acts as a "spacer", increasing the area available to the head group, and leads to the opposite effect; i.e., the head groups can relax into a conformation more parallel to the bilayer surface. These data illustrate the types of steric effects which can be expected at the membrane surface. Dynamic properties were investigated by measurement of the ^2H spin–lattice (T_1) NMR relaxation times. These relaxation times could be compared with those from other parts of a phospholipid molecule, namely, the glycerol backbone and the fatty acyl chains. The rates of segmental motion in these head groups were similar to the first C-2 to C-8 segments of the fatty acyl chains, indicating considerable head-group flexibility.

The conformation and properties of the polar region of a phospholipid, commonly called the "head group", have been a topic of considerable interest. This attention is well deserved, since it is at the level of the head group that the membrane interacts with its environment. This article is the first of a series of three papers concerned with head-group interactions found at the bilayer surface. The approach is founded basically on measurements of the rates of motion of deuterated segments in the various head groups. By comparison of different head groups, the existence and the molecular nature of the various interactions can be deduced.

This paper describes the properties of simple alkyl head groups. There are no *attractive* inter- or intramolecular interactions such as electrostatic (salt) or hydrogen bonds possible between the alkyl portion of the head groups and neighboring head groups (van der Waals interactions will be ignored in this treatment). All of these analogues bear formally one negative charge under the conditions used here and, other than the size of the head group, are absolutely identical. Therefore, these analogues are suitable for a study of steric effects at the membrane surface and provide a logical reference point for comparison with other head groups. In the second paper, hydroxyl groups will be introduced at various positions, and their effects on the motional and ordering properties will be analyzed. Finally, in the third paper, in what is a more complex case, amine groups will be introduced. Head-group interactions have proven to be extremely difficult to study in the relevant bilayer state. Many of the methods applied to this problem have been reviewed (Hauser & Phillips, 1979; Yeagle, 1978; Büldt & Wohlgemuth, 1981).

In this approach, deuterium and phosphorus NMR methods have been employed. Both nuclei have been successfully used in membrane studies [for representative articles on ^2H NMR, see Seelig (1977), Seelig & Seelig (1980), Büldt & Wohlge-

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