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A RELATIONSHIP BETWEEN ANION TRANSPORT AND A STRUCTURAL TRANSITION OF THE HUMAN ERYTHROCYTE MEMBRANE

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Summary

Scanning microcalorimetry was employed as an aid in examining some structural features of the anion transport system in red blood cell vesicles. Two structural transitions were previously shown to be sensitive to several covalent and non-covalent inhibitors of anion transport in red cells. In this study, these transitions were selectively removed, either thermally or enzymatically, and the subsequent effect on $^{35}SO_4^{2-}$ efflux in red cell vesicles was determined. It is shown that removal of one of these transitions (B_2) has a negligible inhibitory effect on anion transport. Cytoplasmic, intermolecular disulfide linkages between band 3 dimers are known to form during the B_2 transition. The integrity of the 4,4′-diisothiocyanostilbene-2,2′-disulfonate-sensitive C transition, on the other hand, is shown to be a requirement for anion transport. The localized region of the membrane giving rise to this transition contains the transmembrane segment of band 3, as well as membrane phospholipids. The calorimetric results suggest a structure of band 3 which involves independent structural domains, and are consistent with the transmembrane segment playing a direct role in the transport process.

Introduction

A great deal of effort has been expended in recent years in studying the anion transport system in red blood cells. The results of labeling whole cells with various specific covalent inhibitors have established that band 3, a trans-

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; imosM, ideal milliosmolar; PMSF, phenylmethylsulfonyl fluoride.

membrane protein of molecular weight 95 000, contains a site capable of regulating transport [1,2]. This inhibitory site is also, quite likely, the transport site for this facilitated diffusion system [3]. Recent kinetic measurements have also established the existence of a modifier site on band 3, distinct from the transport site, which is accessible from the external surface of the cell [24]. Supporting evidence for the direct involvement of band 3 in anion transport has come from recent reconstitution studies [4,5]. It has also been shown that red cell vesicles from which nearly all the membrane proteins except band 3 have been selectively removed retain most of the transport properties of whole cells [6].

We have taken a somewhat different approach in an attempt to elucidate further the anion transport mechanism. Scanning microcalorimetry is known to be a sensitive probe of local membrane structure [7,8]. In a recent study, it was shown that two thermally induced structural transitions in red cells are sensitive to several inhibitors of anion transport [9]. One of these transitions (B_2) is known to involve the cytoplasmic portion of band 3; the other (C) appears to involve phospholipid and also likely the transmembrane portion of band 3. The inhibitors included covalent modifiers of transport such as DIDS which cause a large shift of the C transition only, as well as several reversible inhibitors, which generally shift both the C and B_2 transitions. It was postulated that the local regions of the membrane giving rise to these transitions may be part of the transport system in whole cells.

In this report, we present results of transport studies which demonstrate that changes in transport capabilities in red cell vesicles are correlated only with the loss of the C transition. Thus, it seems likely that the region of the membrane involved in the C transition is indeed part of the anion transport mechanism in red cells. Modifications of the B_2 transition produce little, if any, change in transport properties.

Experimental Procedure

Erythrocyte ghosts were prepared from fresh or recently outdated blood by using the method of Dodge et al. [10]. Vesicles were prepared by alkaline extraction at low salt concentration in either of two ways as follows: Using a procedure similar to that of Steck [11] for the preparation of right-side-out vesicles, 1.0 vol. of freshly prepared ghosts was incubated on ice with 40 vol. of 0.5 mM sodium phosphate, pH 8.0—8.5 for approx. 1 h. Following centrifugation, the ghosts were washed twice in the buffer employed for flux measurements (buffer A: 58 mM Na_2SO_4, 20 mM Tris, 100 mM sorbitol, 2 mM $MgSO_4$, 0.2 mM dithiothreitol, pH 7.4); alternatively, 5 vol. of 2 mM EDTA, 0°C, were added to 1 vol. of packed ghosts and the pH was adjusted to 12. After incubating for 5 min on ice, the pH was readjusted to neutrality prior to centrifugation at 27 000 × *g* for 10 min. The membrane residue was then washed twice in the appropriate buffer. In both cases, the membranes were then sonicated under N_2 in a bath sonicator (55 kHz) for 10 min at 0°C.

External proteolytic digestion of whole cells by pronase was accomplished by dissolving the enzyme (protease, type VI, Sigma) in Tris-saline buffer (140 mM NaCl, 29 mM Tris, pH 7.4) and adding packed cells (20% final hemato-

crit). The suspension was incubated for 1 h at 37°C. The cells were then washed three times in Tris-saline buffer containing 0.5% bovine serum albumin, and finally washed three more times with buffer alone prior to lysis.

External proteolysis by chymotrypsin was accomplished similarly. Thus, the enzyme (Sigma, type II) was dissolved in phosphate-buffered saline (149 mM NaCl, 12 mM sodium phosphate, pH 7.4), added to whole cells (10% hematocrit, 0.4 mg/ml final enzyme concentration), and incubated for 1 h at 37°C. Following the incubation, the suspension was centrifuged and proteolysis was terminated by adding an equal volume of a 5-fold concentration of PMSF in 20% isopropanol/phosphate buffered saline (388 imosM). This was followed by several washes in phosphate-buffered saline prior to lysis.

A modification of the procedure of Steck [12] was employed to generate membranes containing only the transmembrane segment of band 3. Freshly prepared membranes were added to an equal volume of buffer containing 0.2 mg/ml of chymotrypsin. This suspension was incubated at 23°C, with stirring, for 1 h. The suspension was then cooled on ice to 0°C, and the pH was adjusted to 12 with NaOH. After 5 min, the pH was lowered to neutrality and the suspension was centrifuged. Inhibition with PMSF was accomplished as described above.

DIDS was synthesized and reacted with whole cells as described previously [2,9].

Efflux measurements were conducted under equilibrium self-exchange conditions. Typically, 500—600 μCi of $Na_2{}^{35}SO_4$ were added to 0.5 ml of membranes either before or after vesiculation. Sephadex filtration [6,13] was used to remove extracellular radioactivity. The vesicles were cooled to 4°C and loaded on a small column of Sephadex G-75. The column consisted of a 16 × 150 mm test tube fitted with a short length of 3 mm inner diameter tubing at the bottom. Bed volumes for these columns were about 4.5 ml. The vesicles were eluted with cold buffer A and collected on ice. A 1 ml aliquot of this cold suspension was then mixed with an equal volume of buffer A prewarmed to 50°C. The sample was placed in a jacketed vessel maintained at 25°C with constant stirring. At various time intervals, 80-μl samples were withdrawn and placed in small test tubes kept in an ice-water bath. Finally, 50-μl portions were loaded on small columns (2 ml) of cold Sephadex G-75 constructed from Pasteur pipets, the tips of which were drawn out. The vesicles were eluted with exactly 1.2 ml of cold buffer A into scintillation vials. Scintillation fluid (10 ml) was added and the activity counted in a Beckman LS-250 liquid scintillation counter.

Influx measurements were performed by introducing the $^{35}SO_4^{2-}$ into 1.0 ml of a vesicle suspension at 25°C. Samples were withdrawn at various time intervals, loaded on small Sephadex columns, and eluted in a manner identical to that used for efflux measurements. Time constants were calculated from the slopes of semilogarithmic plots of $(y - y_t)/(y - y_0)$ vs. time [25], where y_0, y and y_t represent initial, equilibrium and intermediate values of intracellular radioactivity, respectively. Results are presented as half-times ($t_{1/2}$) for efflux.

Some of the heat capacity measurements were conducted with a specially constructed differential scanning microcalorimeter of high sensitivity [14,

15]. More recent measurements were made on a commercially available instrument (MicroCal Inc., Model MC-1, Amherst, MA).

Results

Preliminary calorimetry and flux measurements

Fig. 1 shows the results of heat capacity measurements on erythrocyte membranes in 310 imosM sodium phosphate, pH 7.4. Each of the five peaks, labeled A—D, is believed to be due to a localized structural transition induced by thermal stress. The origins of these transitions are discussed in detail elsewhere [7—9]. The A transition, for example, is due to a partial unfolding of spectrin. The B transitions are known to involve membrane proteins but may also involve phospholipids as well in an as yet unclear manner.

We have shown previously that the C and B_2 transitions are sensitive to several inhibitors of anion transport in whole cells [9]. Covalent modifiers such as DIDS increase the transition temperature (T_m) of the C transition by as much as 13°C, whereas most of the noncovalent inhibitors depress T_m of either the B or C transition, or of both. The object of this work is to determine whether the C and/or B_2 'regions' of the membrane participate in the transport process in whole cells, as might be expected in view of their sensitivity to anion transport inhibitors.

Fig. 2 shows a heat capacity curve for red cell membranes which had been subjected to prior alkaline extraction (pH 11.5—12, 0°C, 5 min). Only the C and D transitions remain unaltered after such treatment. The A and B_1 transitions, which involve spectrin, and bands 2.1, 4.1 and 4.2, respectively [7,8], are largely missing due to extensive depletion of these peripheral proteins (Ref. 16 and confirmed in this study.) The explanation for the disappearance

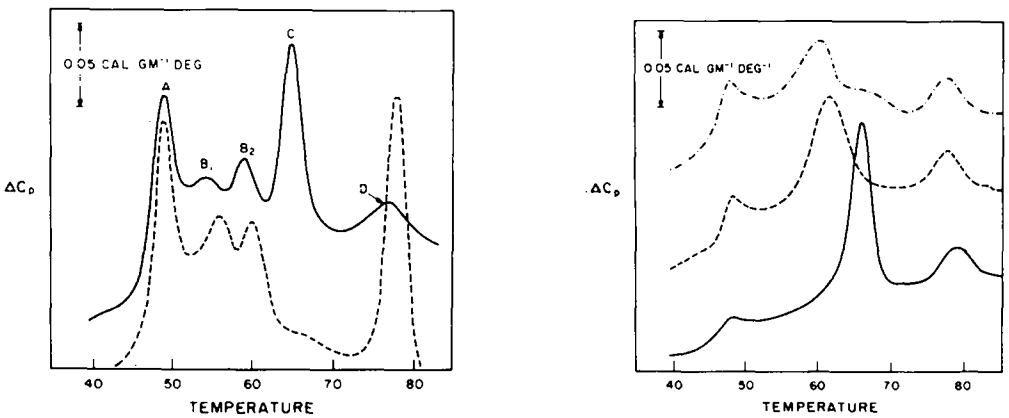

Fig. 1. Heat capacity curves for erythrocyte membranes in 310 imosM sodium phosphate, pH 7.4: (———) unmodified membranes, (- - - - -) DIDS-labeled membranes. Whole cells were reacted with 1 μM DIDS, lysed, washed several times, and resuspended in 310 imosM sodium phosphate.

Fig. 2. Heat capacity for erythrocyte membranes in 310 imosM sodium phosphate, pH 7.4: (———) alkaline-extracted membranes (pH 11.5—12, 0°C, 5 min), (- - - - -) membranes cleaved externally with pronase, 0.8 mg/ml, (· — · — ·) membranes cleaved externally with pronase, 3.9 mg/ml. Pronase-treated membranes were extracted in alkaline medium subsequent to lysis.

TABLE I

Efflux: control samples were subjected to alkaline extraction only. The time dependence of influx was found to exhibit more than one phase when plotted semilogarithmically. This phenomenon, noted previously for both SO_4^{2-} [6] and glucose [17] transport, undoubtedly reflects a heterogeneous population of vesicles, both in size and composition. It was possible to reduce the slower component in efflux measurements by pre-loading the vesicles for short periods of time (2 min); consequently, all subsequent flux data were determined from efflux measurements using short loading times. Influx: the kinetic data for influx measurements were resolved into two exponential phases [18]. The $t_{1/2}$ value for the faster phase is listed here.

Experiment	Calorimetric effect	$t_{1/2}$ (min)
Control (efflux)	No effect on C transition	13.0 ± 1.5
Control (influx)	No effect on C transition	14.3
Whole cells reacted with 100 μM DIDS	C transition shifted from 66 to 78°C	1200
Membranes heated at 1 K/min in buffer A, pH 7.4, to 57°C	Neither B_2 nor C transition effected	10.4
Membranes heated in 310 imosM sodium phosphate, pH 7.4, to 62°C	B_2 region inactivated	12.5
Membranes heated in buffer A, pH 7.4, to 63.5°C	C region partially inactivated	33.0
Membranes heated in buffer A, pH 7.4, to 67.5°C	C region completely inactivated	69.0
Whole cells treated externally with pronase, 0.8 mg/ml	C transition shifted to 62°C and slightly reduced in size	16.8
Whole cells treated externally with pronase, 3.9 mg/ml	C transition shifted to 60°C, further reduced in size	44.7
Whole cells treated externally with α-chymotrypsin, 0.4 mg/ml	No effect on C transition	9.5
Membranes cleaved bilaterally with α-chymotrypsin, followed by alkaline extraction	No effect on C transition	22.7

of the B_2 transition, which involves band 3 as well as phospholipid, is unclear. Mild alkaline extraction of spectrin (pH 8.0—8.5, 0°C, 30 min) leaves all the transitions unchanged except for the A transition, which is reduced to about half of its normal size (not shown).

The results of $^{35}SO_4^{2-}$ measurements are presented as $t_{1/2}$ values in Table I (see Experimental Procedure). Flux measurements were conducted with membrane vesicles subjected to similar extraction procedures. The peripheral proteins do not play a major role in anion transport, and their removal facilitates vesiculation. It has also been shown that further removal of all membrane proteins except band 3 does not appreciably affect transport in vesicles [6].

Several disulfonic stilbene derivatives are known to interact specifically with whole red cells and effectively inhibit anion exchange. The most potent of these inhibitors, DIDS, was reacted with whole cells at a concentration sufficient to produce complete inhibition. The results of efflux measurements on vesicles produced from these inhibited cells, as well as corresponding calorimetric measurements, are shown in Table I and Fig. 1, respectively. The almost complete abolition of SO_4^{2-} flux is convincing evidence that efflux from vesicles prepared as described above is representative of the transport system in whole cells. The calorimetric scan on these vesicles is nearly the same as earlier scans obtained on DIDS-inhibited ghosts, where the C transition is shifted to higher temperature by approx. 13°C. Other evidence also supports the assertion that

the anion transport mechanisms in these two systems are analogous: We have found that the pH dependence of $^{35}SO_4^{2-}$ flux is similar in whole cells and in vesicles; Wolosin et al. [6], using vesicles prepared in a manner similar to ours, demonstrated that the activation energy and anion selectivity in the two systems are similar.

Effects of heating

In order to investigate the possibility that the regions of the membrane giving rise to the C and B_2 transitions may be directly involved in transport, we have utilized the fact that each of the calorimetric transitions shown in Fig. 1 is thermally irreversible. It should then be possible to inactivate selectively the C and/or B_2 regions by heating membranes and/or vesicles through the corresponding transitions. The strong pH dependence of the B_2 transition is helpful in this regard [8]. In 310 imosM sodium phosphate at pH 7.7, the B_2 transition occurs at 56°C, whereas at pH 6.15 it occurs at 72°C. The pH dependence of the C transition is less pronounced and in the opposite direction. Thus, it should be possible to inactivate only the B_2 region of the membrane by heating to about 60°C at a slightly alkaline pH, and to inactivate only the C region by heating to 64°C in slightly acidic buffer. Alternatively, the C transition may be selectively removed by heating to 67°C in buffer A, pH 7.4. In this buffer system, the C transition occurs near 62°C and the B_2 transition is at 69°C (calorimetry not shown). The primary reason for this calorimetric behavior in buffer A is undoubtedly the fairly large negative temperature coefficient of pH for Tris buffer.

Table I shows the results of several heating experiments. Heating the membranes to a temperature just before either the C or B_2 transitions produces no inhibition of SO_4^{2-} flux from vesicles. Similarly, heating through only the B_2 transitions results in a $t_{1/2}$ value equal to that of the control (no heating). Selectively inactivating the C region, on the other hand, produces a marked inhibitory effect. The complete removal of the C transition by heating results in a $t_{1/2}$ value which is over 500% greater than values obtained for unmodified membranes. Heating to a point halfway through the C transition produces a $t_{1/2}$ value approximately half that obtained by heating completely through the peak.

Effects of enzymatic modification

The use of proteolytic enzymes has been a valuable tool in learning about the anion transport mechanism in whole blood cells. Externally applied pronase, for example, has been shown to cleave band 3 and inhibit SO_4^{2-} flux in whole cells [18,19] and to decrease the activation energy [20]. Loss in transport capability was correlated with a loss in H_2DIDS binding sites on band 3. External chymotrypsin, on the other hand, has little effect on either SO_4^{2-} transport or total H_2DIDS binding to band 3 [19] even though cleavage of band 3 takes place.

It is informative to add these enzymes to suspensions of whole cells under the conditions described above and subsequently to determine the effects on calorimetry and SO_4^{2-} efflux from the corresponding vesicles. Fig. 3 shows the calorimetric scan which results from externally applied chymo-

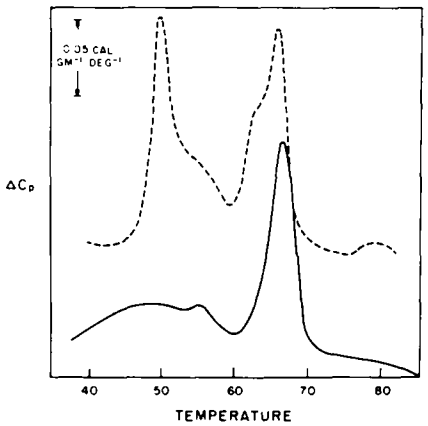

Fig. 3. Heat capacity curves for erythrocyte membranes in 310 imosM sodium phosphate, pH 7.4: (———) membranes were subjected to bilateral proteolysis with α-chymotrypsin, followed by alkaline extraction, (- - - - -) membranes subjected to external chymotryptic cleavage.

trypsin. The A, C and D transitions are unaltered. The B_1 and B_2 transitions also appear to be largely intact, except they are no longer as well resolved from the A and C transitions, respectively. This is probably due to the exposure to 10% isopropanol during inhibition with PMSF since an identical control, which lacked only chymotrypsin, exhibited a similar effect (not shown). The corresponding kinetic parameter (Table I) indicates that no inhibition of transport has occurred.

The failure of external chymotrypsin to affect either the B_2 or the C transition, or to inhibit transport, is in contrast to the results obtained by external cleavage with pronase. Fig. 2 demonstrates that increasing concentrations of pronase depress the T_m of the transition and also reduce ΔH. These modifications in the C transition are correlated with increasing inhibition of SO_4^{2-} efflux (Table I). Again, the A and B transitions are eliminated in Fig. 2 due to the alkaline extraction employed during the vesiculation procedures. We have determined previously that neither external pronase nor chymotrypsin has a substantial effect on the A and B transitions when extraction and vesiculation are avoided.

It is of interest to note that the enthalpy changes, ΔH, during the C transition for the control and the two pronase-treated samples have the proportions, in order of increasing enzyme concentration, 1.0 : 0.84 : 0.24. From Table I, the corresponding values of the reciprocal half-time decrease in the proportion 1.0 : 0.77 : 0.29. These results, together with those from the heating experiments, suggest that the loss in transport capability in red cell vesicles is nearly proportional to the decrease in ΔH for the C transition.

A combination of bilateral proteolytic digestion by chymotrypsin followed by alkaline stripping removes all the peripheral proteins and leaves only the 17 kdalton transmembrane segment of band 3 and smaller segments of other integral proteins (see Experimental Procedure) within the membrane, as confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The effects of such treatment on calorimetry and transport are shown in Fig. 3

and Table I, respectively. The C transition is the only structural transition left unaltered by this procedure. Only a relatively small degree of inhibition occurs as a result of this procedure, as shown in Table I. A similar small inhibitory effect was produced by bilateral cleavage with papain.

Discussion

In a previous publication [9] we presented calorimetric data which showed that the C transition was strongly shifted to higher temperature by the addition of low concentrations of covalent inhibitors of anion transport such as DIDS, SITS and pyridoxal phosphate. Several non-covalent inhibitors of anion flux were found to produce downward shifts in the B_2 transition, as well as in the C transition in some cases. Thus, it seemed possible that the regions of the membrane giving rise to both of these transitions could be part of the transport machinery. However, a certain amount of caution was necessary in the interpretation of the data with non-covalent inhibitors, since most of these amphipathic molecules are known to be fairly non-specific agents which can generally be absorbed into much of the lipid region of a membrane, and are known to shift structural transitions in phospholipid vesicles.

The present results show that the thermal disruption of the region of the membrane giving rise to the B_2 transition does not produce any significant alteration in the ability of the membrane to transport SO_4^{2-}. This observation is also reinforced by other findings. For example, data in this paper show that alkaline (pH 12) extraction abolishes the B_2 transition, but that vesicles formed from these extracted membranes have a transport capacity similar to that of vesicles formed from membranes which still retain the B_2 transition. Also, bilateral proteolysis with papain, followed by alkaline extraction, removes all membrane peripheral proteins and reduces band 3 to a 17 kdalton transmembrane fragment. Bilateral cleavage with α-chymotrypsin produces a similar effect. These procedures completely abolish the B_2 transition (as well as the A and B_1 transitions) but leave the C transition unchanged. In spite of the complete loss of the B_2 transition, vesicles formed from membranes treated in this manner show only moderately impaired transport capability. Ramjeesingh et al. [26] have also recently noted that bilateral chymotryptic cleavage of leaky ghosts produces some inhibitory effect on anion transport capability in red cell membranes. In this case, the inhibitory effect was associated with additional external cleavages which (apparently) do not occur in whole cells.

Thus, it appears that the B_2 region of the membrane is not involved directly in anion transport. There is much evidence now which suggests that band 3 is an important participant in the B_2 transition. Most importantly, it has been shown [29] that band 3 molecules associate covalently by disulfide bonds to form dimers over the narrow temperature region of the B_2 transition (further aggregation occurs at higher temperature). The interactions appear to be confined to the cytoplasmic portion of the molecule, however. Since band 3 is believed to exist as a non-covalent dimer under physiological conditions [12], it seems reasonable that the disulfide linkages probably occur between these nearest neighbors. External chymotrypsin cleaves band 3 to a 60 kdalton membrane-attached fragment, but has no effect on the B_2 transition (or trans-

port), while additional cleavage of the cytoplasm portion of band 3 with trypsin or chymotrypsin causes the complete loss of the transition.

Thermal and enzymatic modifications of the C transition with pronase, on the other hand, produce marked inhibitory effects on SO_4^{2-} exchange. The rate constant for efflux from vesicles with thermally inactivated C regions was less than 20% of rate constants for unmodified membranes. Partial removal of the C transition by either thermal or enzymatic modification resulted in partial inhibition. Based on $t_{1/2}$ values in Table I, the partial inhibition is roughly proportional to the loss in ΔH of the C transition. These findings provide strong evidence for the necessity of having an intact C region of the membrane in order to function normally in anion transport. They are in agreement with our previous experimental findings on the effects of external DIDS binding; namely that the DIDS-induced shift of the C transition to higher temperatures correlates with the inhibitory effect on SO_4^{2-} efflux [9].

Because of its sensitivity to DIDS binding and of its necessity in anion transport, the C region of the membrane must contain or be strongly influenced by portions of band 3. However, most of band 3 is probably not directly involved in the transition, since extensive bilateral digestion by chymotrypsin (or papain), which cleaves band 3 to a 17 kdalton transmembrane fragment, has no measurable effect on the C transition. Furthermore, recent reconstitution studies have demonstrated that incorporation of the transmembrane fragment of band 3 in phosphatidylserine/phosphatidylcholine vesicles produces a calorimetric transition near 55°C. If the 17 kdalton fragment is derived from DIDS-treated cells, this transition is shifted upwards by more than 10°C. (Low, P., personal communication).

Along with the transmembrane fragment of band 3, membrane phospholipids have been shown to be required for an intact, functioning C region. Thus, the action of both phospholipase A_2 and C has been shown to lower the temperature of the C transition, to decrease its size, and to inhibit strongly anion transport in the process. Also, a number of solutes known to affect lipid transitions in synthetic vesicles cause pronounced shifts of the C transition [8,9,22].

Although the precise molecular events which occur in the C transition are not yet known, several pertinent observations have been made. For example, no circular dichroism change was found to occur [7] in the C transition in the peptide-sensitive region (223 nm). However, large changes in light scattering have been observed, and freeze-fracture electron microscopy studies show that strong irreversible aggregation of the 80 Å particles takes place in the temperature region of the C transition (unpublished observations).

Although external proteolysis with most proteases has no effect on the C transition or on anion transport capability, this is not true for pronase. High concentrations of pronase act to inhibit anion transport and to lower the temperature and decrease the size of the C transition. Evidently, pronase is capable of cleaving the band 3 chain closer to the transmembrane segment and thereby interfering with the C domain and with anion transport.

In summary, the results presented in this paper are consistent with the idea that the integrity of the C region (the region of the membrane giving rise to the C transition) is a necessary, although perhaps not sufficient, condition for

anion transport functionality in erythrocyte membranes. Several lines of evidence have been presented which demonstrate roles for the transmembrane fragment of band 3 and membrane phospholipid in this region.

The calorimetric results give additional insight about structural features of the transport machinery. Thus, it is interesting to note that, while the B_2 and C transitions both involve band 3, different domains of this polypeptide participate in the two transitions; namely, the B_2 transition involves the cytoplasmic portion of band 3, whereas the C transition involves the transmembrane segment. The extracellular portion can be cleaved off enzymatically with chymotrypsin, with no effect on either B_2 or C. Band 3, therefore, must be comprised of three (or more) structurally independent domains. Although the anion transport site lies within the transmembrane domain of band 3, the cytoplasmic and exterior portions could possibly play some regulatory role in the transport process. Consistent with this idea, it is known that several other important erythrocyte proteins, including oxyhemoglobin [23], glyceraldehyde-3-phosphate dehydrogenase [27] and aldolase [28] bind reversibly to the cytoplasmic domain of band 3. Also, kinetic studies have demonstrated the existence of a modifier site, distinct from the transport site, and accessible only from the exterior of the membrane [24].

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