Evaluation of Structural Interdependence of Membrane-Spanning and Cytoplasmic Domains of Band 3†

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ABSTRACT: The structural interdependence of the membrane-spanning (55 000 dalton) and cytoplasmic (40 000 dalton) domains of the erythrocyte membrane protein band 3 was investigated. The influence of the membrane-spanning domain on the behavior of the cytoplasmic domain was initially examined by comparing the structural properties of the cytoplasmic domain in situ with its properties in isolated form, i.e., after proteolytic removal from the membrane. The previously described calorimetric and fluorescence properties of the isolated cytoplasmic domain [Appell, K. C., & Low, P. S. (1981) *J. Biol. Chem. 256*, 11104–11111] were used as the basis for this comparison. The pH titration of the domain's intrinsic fluorescence was essentially identical before and after cleavage from the membrane. The thermal denaturation temperatures (T_m) of the uncleaved and the isolated cytoplasmic domains were also very similar, decreasing more than 15 °C between pH 6 and pH 8.5. However, the cytoplasmic domain in situ was slightly less stable at all pHs than its isolated counterpart. In contrast, the thermal stability of the membrane-spanning domain of band 3 was essentially unaltered by proteolytic removal of the cytoplasmic domain. These observations suggest that the structures of the membrane-spanning and the cytoplasmic domains of band 3 are not significantly altered by separation after proteolytic cleavage. The effect of denaturation of one domain of band 3 on the thermal stability of the other was also investigated. At pH 6.5, the integral domain denatures at a lower temperature than the cytoplasmic domain. However, in membranes prepared from 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)-labeled cells, the integral domain is stabilized so that it unfolds only after the thermal transition of the cytoplasmic domain. Regardless of whether the integral domain is native or denatured at the onset of thermal unfolding of the cytoplasmic domain, the calorimetric behavior of the latter remains unaltered. Furthermore, by choosing the appropriate membrane stripping procedure (i.e., NaOH or acetic acid), it is possible to selectively denature one of the two major domains of band 3 without significantly perturbing the other. Taken together, the above observations suggest that the extent of interaction between the two major domains of band 3 is minimal.

In a previous article (Appell & Low, 1981) we demonstrated that the cytoplasmic region of band 3 (M_r ~40 000) is released in a highly folded, dimeric state by chymotrypsin digestion of inside-out erythrocyte membrane vesicles. Other researchers have shown that the isolated cytoplasmic fragment of band 3 retains its ability to bind ankyrin (Bennett & Stenbuck, 1980; Hargreaves et al., 1980), glyceraldehyde-3-phosphate dehydrogenase (Yu & Steck, 1975; Steck et al., 1976), aldolase (Strapazon & Steck, 1977), and hemoglobin (Salhany et al., 1980). Since the membrane-spanning fragment (M_r ~55 000) of the anion transport protein also remains functional following release of the cytoplasmic pole (Rice & Steck, 1977; Grinstein et al., 1978), it seems reasonable to propose that the above proteolytically defined regions represent distinct structural domains of intact band 3 (Snow et al., 1981).

The degree of interaction between the membrane-spanning and cytoplasmic domains of band 3 is still a matter of conjecture. Salhany et al. (1980) report that DIDS[1] binding to the integral domain of band 3 alters the binding isotherm of hemoglobin to the cytoplasmic domain. Hsu & Morrison (1981) show that DIDS-labeled membranes retain more ankyrin and spectrin on their cytoplasmic surfaces than their unlabeled counterparts. Rice & Steck (1977) further demonstrate that disulfide cross-linking of the cytoplasmic domain alters pyruvate transport, presumably through the integral domain. These observations would seem to suggest that a structural perturbation in one domain of band 3 will be communicated to the other, i.e., that a measurable direct or indirect interaction between the two domains of band 3 exists. On the other hand, binding of antibodies, glyceraldehyde-3-phosphate dehydrogenase, and aldolase to the cytoplasmic domain of band 3 appears to exert no effect on anion exchange through the integral domain (Rice & Steck, 1977; Steck, 1978; Ross & McConnell, 1978; England et al., 1980). Also, complete proteolytic removal of the cytoplasmic domain from the membrane causes no significant change in anion transport properties (Rice & Steck, 1977; Grinstein et al., 1978), although a small decrease in the transport rate has been reported (Lepke & Passow, 1976). Furthermore, glyceraldehyde-3-phosphate dehydrogenase associated with the cytoplasmic domain appears to exhibit motional freedom relative to the membrane-spanning domain (Beth et al., 1981). Since the cytoplasmic domain shows little tendency to remain associated with the membrane after cleavage, one is tempted to conclude that measurable interactions do not exist between the two domains of band 3.

We have decided to assess the structural independence of the membrane-spanning and cytoplasmic domains of band 3 directly by seeking answers to the following two questions: (1) does each domain behave the same structurally both before and after separation by proteolytic cleavage and (2) do the two structural domains denature independently of each other; that is, can either domain sense the structural integrity of the other? On the basis of both calorimetric and fluorescence

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[1] Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; PMSF, phenylmethanesulfonyl fluoride; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; IOV's, inside-out vesicles depleted of spectrin and actin; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PAS, periodic acid–Schiff base; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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observations we find that the interaction between the two structural domains of band 3 is either minimal or nonexistent. However, some interaction between the cytoplasmic domain and other components of spectrin-depleted, KCl-stripped membranes cannot be ruled out.

Experimental Procedures

Materials

α-Chymotrypsin (65 units/mg), phenylmethanesulfonyl fluoride (PMSF), 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid (Tes), and egg phosphatidylcholine were purchased from Sigma Chemical Co. DEAE-Affi-Gel Blue was from Bio-Rad and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was obtained from Pierce Chemical Co.

Methods

Preparation of Samples for Differential Scanning Calorimetry. The 40 000-dalton cytoplasmic fragment of band 3 was cleaved from erythrocyte membranes and isolated as described previously (Appell & Low, 1981). The various membrane samples were prepared as outlined in the figure legends and then washed twice in the appropriate buffer, preadjusted to the desired pH. When Tes buffer was used, the solution pH at the temperature of each transition was calculated by assuming a $\Delta pK_a/°C$ of −0.02 (Weast, 1977).

Heat capacity measurements were obtained on a Microcal 1 differential scanning calorimeter (Amherst, MA) equipped with matched 1-mL platinum cells. Membrane samples equilibrated in the desired buffer were loaded into the sample cell, and an equal volume of the identical buffer system was added to the reference cell of the calorimeter. The heating rate in these experiments was 1 °C/min. The membrane concentrations used in the calorimeter were determined by drying the samples to a constant weight at 96 °C, and these concentrations were normally about 1% by weight.

Because the base-line slope of the calorimeter scan was not always horizontal, the midpoint temperature (T_m) of each calorimetric transition was taken as the temperature where the vertical distance between the linear base line and the heat capacity curve was maximal. The midpoint temperature is therefore the temperature of maximum excess heat capacity. When the base-line slope is strongly positive, this temperature will lie slightly (generally <1 °C) below the temperature of maximum vertical pen displacement. By using this procedure, the T_m values of a particular transition under different experimental conditions were compared.

Intrinsic Fluorescence of Inside-Out Vesicles. The intrinsic fluorescence of IOV's, prepared according to Steck et al. (1976), was monitored in the ratio mode on a Perkin-Elmer MPF-44A fluorescence spectrophotometer equipped with two thermostated quartz cuvettes. The excitation and emission monochromators were set at 290 and 333 nm, respectively, and the corresponding slits were adjusted to a spectral bandwidth of 4 nm. Membrane samples were prepared for observation by diluting 75 μL of a membrane stock solution into 2.5 mL of 50 mM sodium phosphate, 50 mM sodium borate, and 70 mM NaCl, preadjusted to the desired pH. The intrinsic fluorescence of inside-out vesicles depleted of spectrin and actin was compared with the intrinsic fluorescence of the same vesicle preparation after digestion with α-chymotrypsin. The proteolysis was found to remove the cytoplasmic domain of band 3. Both membrane samples were washed twice in the borate/phosphate buffer at pH 7.5 prior to dilution.

Analytical Procedures. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Fairbanks et al. (1971) with 5.6% disc gels in a buffer that contained 0.2% sodium dodecyl sulfate. Protein concentrations were obtained by using the procedure of Lowry et al. (1951). pH titration curves were analyzed on a Tektronix 4051 computer with a nonlinear least-squares program developed by W. R. Finkenstadt at Purdue University.

Results

Identification of the Thermal Denaturation Transition of the Cytoplasmic Domain of Band 3 in Situ. In a previous paper (Appell & Low, 1981) we described several calorimetric and fluorescence properties of the isolated cytoplasmic fragment (M_r ~40 000) of band 3. In order to compare the calorimetric properties of this fragment with the calorimetric properties of the intact cytoplasmic domain in situ (i.e., uncleaved and on the membrane), it was necessary to identify the calorimetric transition of the cytoplasmic domain in whole erythrocyte membranes. Our procedure for assigning this transition to one of the red cell membrane's endotherms can be summarized as follows. Membranes were gradually depleted of their component proteins, and the resulting depleted membrane pellets were scanned in the calorimeter. The denaturation transition of this region of the transport protein was to be identified (vide infra) by observing which transition disappeared upon selective proteolysis of the cytoplasmic domain of band 3.

The scan of intact erythrocyte membranes in Tes-buffered saline, pH 7.0 at 23 °C, is shown in Figure 1B (top scan). Five major endotherms are observed, which presumably correspond to order → disorder transitions in major structural regions of the membrane. The A transition has been shown previously to involve the denaturation of spectrin (Brandts et al., 1977). In agreement with this identification, depletion of spectrin and actin from erythrocyte membranes (Figure 1A, gel B) leads to a loss of the A transition (Figure 1B, second scan). Further removal of bands 2.1, 4.1, and 6 from the membranes by stripping with 1 M KCl (gel C) greatly reduces the size of the B_1 transition but leaves the B_2, C, and D endotherms largely unaltered (middle scan). Since band 3 is unmodified by this treatment, the transport protein can be largely dismissed as an important contributor to the B_1 endotherm. However, mild digestion of the KCl-stripped, inside-out vesicles with α-chymotrypsin causes loss of the residual B_1 transition and removal of much of the B_2 transition (Figure 1B, second scan from bottom).[2] This process reduces most of band 3 to a highly diffuse (Markowitz & Marchesi, 1981) 55 000-dalton membrane-spanning fragment (gel D) and releases the water-soluble cytoplasmic fragment of band 3 into the supernatant (gel E). Both the concentrated supernatant and the isolated cytoplasmic fragment exhibit an endotherm similar to the B_2 transition that was lost upon proteolytic digestion of the membranes (Figure 1B, bottom scan). Thus, the loss of the B_2 transition upon removal of the cytoplasmic domain of band 3 and the concomitant appearance of a similar transition in the cytoplasmic domain extract both indicate the involvement of the cytoplasmic domain in "B_2".

During preparation of the manuscript, a thermal gel analysis of human erythrocyte membranes appeared in press and reported the same conclusion (Lysko et al., 1981). The unfolding temperatures of the major erythrocyte membrane proteins were determined by observing the temperature at which nonnative disulfide bonds formed in each membrane component. The cytoplasmic domain of band 3 was found to undergo random

[2] The extent to which the B_2 transition is removed by mild chymotryptic digestion cannot be accurately assessed because of the overlapping C and D transitions.

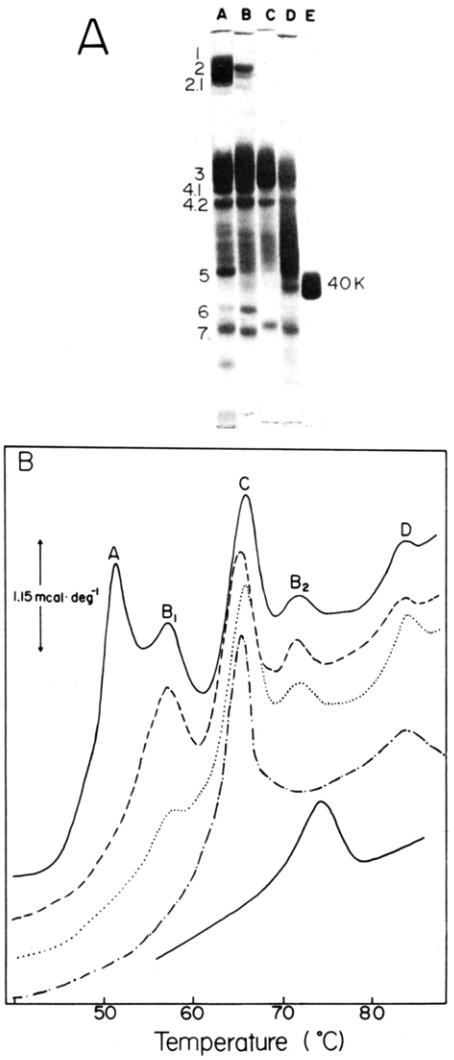

FIGURE 1: (A) $NaDodSO_4$–polyacrylamide disc gels of the membrane and protein samples used in the calorimetric experiments described in (B). Erythrocyte ghosts (gel A) were prepared from 200 mL of fresh human blood by hypotonic lysis in 5 mM sodium phosphate, pH 7.4, as described by Dodge et al. (1963). Membranes depleted of spectrin and actin (gel B) and converted to inside-out vesicles (IOV's) were prepared by incubating the ghosts at 37 °C for 30 min in 10 volumes of 0.5 mM EDTA, pH 8.0 (Marchesi et al., 1970; Steck et al., 1976). Incubation of the IOV's in 25 mM sodium phosphate, 25 mM EDTA, and 1 M KCl, pH 8.5, for 30 min at 37 °C (Tyler et al., 1979) led to the further release of bands 2.1, 4.1, and 6 (gel C). The 40 000-dalton cytoplasmic fragment of band 3 was then cleaved from the vesicles by washing with 10 mM sodium phosphate, pH 7.5, and then suspending in 20 volumes of the wash buffer containing 1 μg/mL α-chymotrypsin. After incubation at 1 °C for 45 min, the digestion was terminated by addition of PMSF to a final concentration of 200 μg/mL. The pellet (gel D) was removed by centrifugation, and the cytoplasmic fragment (gel E) was isolated as described previously (Appell & Low, 1981). The electrophoresis was conducted essentially as described by Fairbanks et al. (1971). (B) The heat capacity as a function of temperature of the membrane/protein preparations described in (A). The calorimetric scans were conducted at 1 °C/min on 1-mL samples equilibrated in 20 mM Tes and 125 mM NaCl, pH 7.0, at 23 °C. Erythrocyte ghosts (11.91 mg of protein) (—) were depleted of spectrin and actin (9.23 mg of protein) (---), stripped with 1 M KCl (7.98 mg of protein) (···), and mildly digested with α-chymotrypsin (6.45 mg of protein) (-·-), as described in the legend to (A). The purified cytoplasmic fragment (—) was adjusted to a protein concentration of 1.60 mg/mL and dialyzed against the above Tes buffer. The $\Delta pK_a/°C$ of Tes buffer is −0.02, so that the pH of the fragment preparation at 74 °C is ~6.0.

disulfide cross-linking at the temperature of the B_2 transition, even if the B_2 transition was shifted to higher or lower temperatures by changing the pH. Thus, the involvement of the

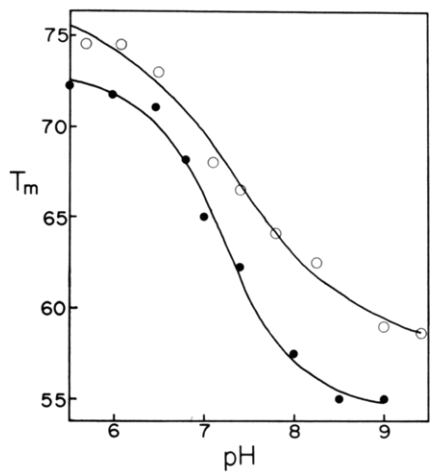

FIGURE 2: Temperature of maximum heat capacity (T_m) of the B_2 transition and the isolated cytoplasmic fragment of band 3 plotted as a function of pH. Spectrin-depleted membranes were stripped with 1 M KCl and washed twice in 0.05 M sodium phosphate, 0.05 M sodium borate, and 0.07 M NaCl, preadjusted to the desired pH, and then scanned in the calorimeter. The data on the isolated cytoplasmic fragment were obtained from Appell & Low (1981). The nonlinear least-squares best fit of the data to the Hill equation (—) is shown with the experimentally obtained data points for the isolated 40 000-dalton cytoplasmic fragment (○) and the B_2 transition (●).

cytoplasmic domain of band 3 in the B_2 transition seems assured.

Comparison of the Properties of the Cytoplasmic Domain in Situ and in Isolated Form. With the calorimetric transition of the cytoplasmic domain of band 3 now identified, it is possible to compare the behavior of the cytoplasmic pole of band 3 before and after its cleavage from the membrane. The rationale for this comparison is as follows: if significant differences in the properties of the membrane bound and free forms of the cytoplasmic domain are observed, then substantial interaction between the domain and the membrane will be implicated. However, if the properties of the bound and cleaved forms are highly similar, then little or no membrane interaction can be claimed.

One of the most peculiar properties of the isolated cytoplasmic fragment of band 3 is its exquisite sensitivity to pH (Appell & Low, 1981). Thus, the fragment denatures[3] at ~74 °C at pH 6 but at ~59 °C at pH 9. A direct comparison of the pH dependence of the thermal stabilities of the bound and free forms of the cytoplasmic domain is shown in Figure 2. The data for the isolated fragment are taken from Appell & Low (1981). The T_m of the cytoplasmic domain in situ was obtained under identical solution conditions on KCl-stripped IOV's. The stripping procedure was found to have no effect on the T_m of the B_2 transition but was necessary to remove the B_1 transition, which interferes with the observation of the B_2 transition at high pH. The use of identical solution conditions is essential, since the B_2 transition is very sensitive to ionic strength (Brandts et al., 1978). The earlier titration of B_2 by Brandts et al. (1978) probably differs somewhat from our data for this reason. Both curves in Figure 2 were analyzed by a nonlinear least-squares computer program to obtain the best fit of the data to the Hill equation. The derived apparent pK_a, Hill coefficient, and midpoint temperature of the B_2 transition (7.2, 1.0, and 63 °C, respectively) are very similar

[3] In a previous article (Appell & Low, 1981) the endothermic transition of the cytoplasmic fragment of band 3 was shown to be irreversible, thus suggesting that it derives from protein denaturation rather than a native → native conformational transition.

to but not identical with the same parameters of the isolated fragment (7.3, 0.6, and 66 °C, respectively). The most striking difference in the domain's behavior in the two environments is its thermal stability. Thus, over the entire physiological pH range the uncleaved form is slightly less stable than its isolated counterpart. This suggests that release of the cytoplasmic pole from the membranes releaves a small, but measurable, strain within the cytoplasmic domain. Less significant differences were observed in the other two derived parameters. The low Hill coefficients both support the earlier contention that a single ionizable group is responsible for the observed sensitivity to pH (Appell & Low, 1981). The close similarity in apparent pK_a values also suggests that the environment of the titratable group does not change significantly upon release of the cytoplasmic fragment from the membrane.

A second unusual characteristic of the isolated cytoplasmic domain of band 3 is the sensitivity of the fragment's intrinsic fluorescence to pH (Appell & Low, 1981). Thus, the magnitude of the fluorescence emission at 335 nm (λ_{ex} 290 nm) was found to more than double between pH 6 and pH 10. The temperature-dependent apparent pK_a of the isolated fragment was found to be ~8.1 at 20 °C ($\Delta pK_a/°C \sim -0.018$) and the Hill coefficient was less than one. In order to measure the pH dependence of the intrinsic fluorescence of the cytoplasmic domain in situ, we monitored the difference in fluorescence intensity between IOV's lacking the cytoplasmic domain (due to chymotryptic cleavage) and those with band 3 intact. For purposes of comparison, the concentrations of the two preparations were adjusted to obtain the same intrinsic fluorescence at pH 6. Figure 3A shows the fluorescence intensity of the two IOV preparations as a function of pH at 23 °C. As pH is raised, the fluorescence of the uncleaved preparation increases relative to the fluorescence of the mildly digested preparation. In order to focus directly on the behavior of the cytoplasmic domain in situ, we subtracted the two curves and plotted the difference in Figure 3B. The calculated apparent pK_a of 8.1 at 23 °C and the Hill coefficient of 0.7 both agree closely with the values obtained with the isolated fragment (vide supra). Thus, despite the obvious approximations inherent in the above measurements, the isolated fragment of band 3 appears to respond to pH in a manner similar to the cytoplasmic domain in situ.

Evaluation of the Independence of the Denaturation Transitions of the Integral and Cytoplasmic Domains of Band 3. One of the most stringent tests of an independent hypothesis is to determine if the two putative domains can denature independently of each other. We decided to conduct this test on band 3. The thermal unfolding of the cytoplasmic domain of band 3 was shown above to occur during the B_2 transition. The thermal denaturation of the membrane-spanning domain of band 3 has been recently shown to occur during the prominent C transition (Davio & Low, 1982). The most compelling evidence for this conclusion is obtained from reconstitution studies. In these experiments the purified membrane-spanning fragment of band 3 was found to yield a relatively unaltered C transition upon reconstitution into egg phosphatidylcholine/bovine brain phosphatidylserine vesicles. Other aspects of this work demonstrate that the structural change in band 3 during the C transition is substantial, suggesting that the C endotherm derives from the actual denaturation of the membrane-spanning domain.

With the calorimetric transitions of both domains of band 3 located, we can ask whether denaturation of one region of band 3 alters the stability of the other. To evaluate this question, we have analyzed the behavior of the B_2 transition

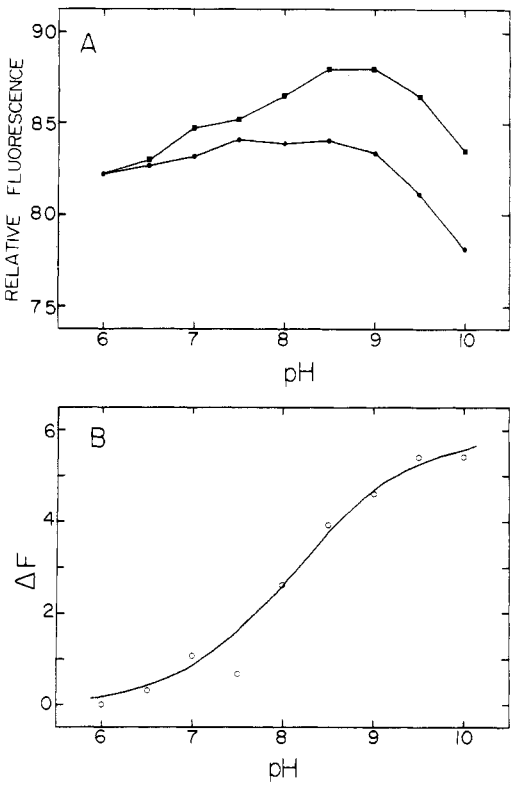

FIGURE 3: (A) Fluorescence intensity of spectrin-depleted inside-out erythrocyte membrane vesicles before (■) and after (●) removal of the cytoplasmic domain of band 3 by chymotryptic cleavage. Membrane samples were dissolved in 50 mM sodium phosphate, 50 mM sodium borate, and 0.07 M NaCl, preadjusted to the desired pH. The protein concentrations for the undigested (27 μg/mL) and digested vesicles (33 μg/mL) were adjusted to obtain the same intrinsic fluorescence at pH 6. The relative magnitude of the fluorescence emission at 333 nm (λ_{ex} 290 nm) is plotted as a function of pH at 23 °C. (B) The difference in fluorescence intensity (ΔF) between the above two titration curves in part A. The solid line represents the nonlinear least-squares best fit of the data to the Hill equation.

under conditions where the C transition occurs either prior to or following B_2. At pH 6.5, the B_2 and C transitions are centered at ~72 and 66 °C, respectively (Figure 4). However, if the erythrocytes are labeled with the covalent transport inhibitor DIDS prior to membrane preparation, the C transition is shifted up ~10 °C along the temperature axis.[4] In these DIDS-labeled membranes, the integral region of band 3 is intact during the thermal disruption of the cytoplasmic domain. In the absence of DIDS, however, the integral domain has already denatured when the cytoplasmic region begins to unfold. Clearly, the structural state of the integral domain has no effect on the calorimetric behavior of the cytoplasmic domain (Figure 4). The cytoplasmic domain, therefore, must be unable to sense the conformation of the membrane-spanning region of band 3.

The structural independence of the two domains of band 3 can be further emphasized by the response of the B_2 and C transitions to various membrane-stripping procedures (Figure 5). Treatment of spectrin-depleted IOV's with 1 M KCl causes removal of the B_1 transition without affecting either B_2 or C transitions. However, incubation of the IOV's with cold 0.01 N NaOH substantially reduces B_2 but diminishes C only slightly (Figure 5). On the other hand, stripping of

[4] The calorimetric scan of the DIDS-labeled membranes has a steeper base-line slope than that observed in the control scan. Such changes in slope are not uncommon between scans and may derive from small differences in the solution composition or volume of the material in the reference and sample cells of the calorimeter.

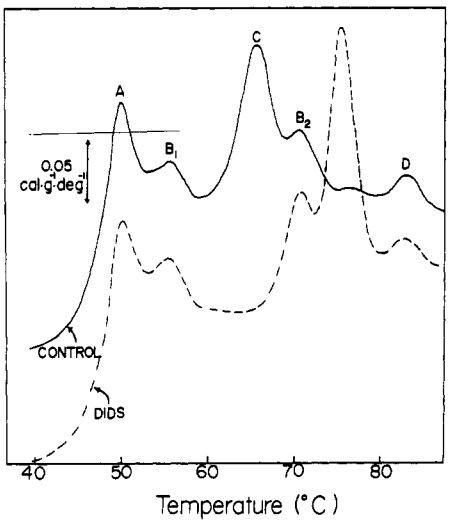

FIGURE 4: Heat capacity of untreated (—) and DIDS-labeled (---) erythrocyte membranes as a function of temperature. The DIDS-labeled membranes were prepared by incubating whole, washed erythrocytes at 10% hematocrit with a 10 μM solution of DIDS for 1 h at 37 °C (Cabantchik & Rothstein, 1974). The cells were washed 2 times in isotonic phosphate buffer, pH 7.4, containing 1% BSA and then 3 times in buffer alone. DIDS-labeled membranes were then prepared by hypotonic lysis. Both the untreated and DIDS-labeled membranes were washed twice in 20 mM sodium phosphate and 125 mM NaCl, pH 6.51, before calorimetry. Under these solution conditions the B_2 transition is centered at ~71 °C.

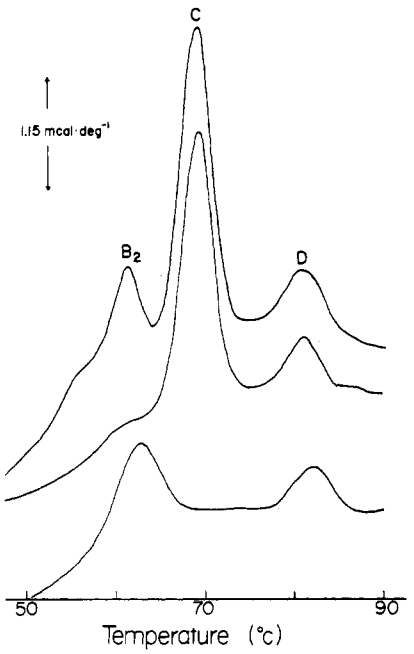

FIGURE 5: Effect of various stripping procedures on calorimetric scans of erythrocyte membranes. Membrane samples were treated with 1 M KCl as described in Figure 1 (upper curve) or with cold 0.01 N NaOH as described by Steck & Yu (1973) (middle curve) or with 0.17 M acetic acid as described by Bennett & Stenbuck (1980) (lower curve) and then washed twice in 20 mM sodium phosphate and 125 mM NaCl, pH 7.5. At this pH the B_2 transition is centered at ~61 °C. The total protein contents of the KCl-, NaOH-, and acetic acid stripped membranes were 8.0, 6.3, and 9.1 mg/mL, respectively.

the IOV's with 0.17 M acetic acid preserves B_2 but destroys C. Thus, by choosing the proper stripping conditions, it is possible to selectively disrupt either the integral or the cytoplasmic domain of band 3, leaving the other domain relatively unaltered.

This same difference in sensitivity to the various membrane-stripping procedures is also manifested by the isolated

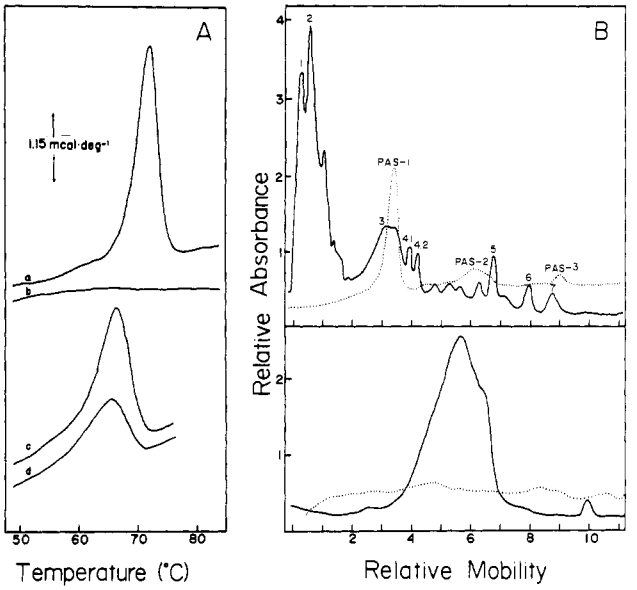

FIGURE 6: (A) Heat capacity as a function of temperature of purified, reconstituted, DIDS-labeled, 55 000-dalton integral fragment (scans a and b) and cytoplasmic fragment (scans c and d) of band 3. Egg phosphatidylcholine liposomes containing the purified 55 000-dalton fragment were incubated in either PBS, pH 7.5 (scan a), or 0.17 M acetic acid (scan b) and then washed twice in PBS, pH 7.5, before calorimetry. The protein contents of the samples studied in scans a and b were 3.98 and 3.83 mg/mL, respectively. The purified cytoplasmic fragment in 10 mM sodium phosphate was incubated for 30 min on ice at pH 7.5 (scan c) or at pH 12 (by addition of 1 N NaOH, scan d). After incubation, both preparations were adjusted to 20 mM sodium phosphate and 125 mM NaCl, pH 7.5, by addition of the appropriate reagents. The control (4.44 mg of protein/mL) and the NaOH-treated (4.40 mg of protein/mL) samples were then scanned in the calorimeter. (B) Densitometric scans of $NaDodSO_4$–polyacrylamide (5.6%) disc gels of total erythrocyte membrane proteins (upper panel) and the purified 55 000-dalton integral fragment of band 3 (lower panel). The 55 000-dalton fragment was prepared essentially as described by Davio & Low (1982) and then incorporated into egg phosphatidylcholine vesicles. The densitometric scans were obtained at 560 nm for both the Coomassie blue stained gels (—) and the periodic acid–Schiff base stained gels (···).

cytoplasmic and membrane-spanning fragments of band 3. Figure 6A (top scan) shows the calorimetric scan of the DIDS-labeled, membrane-spanning fragment of band 3, purified and reconstituted into egg phosphatidylcholine liposomes according to the procedures of Davio & Low (1982).[5] The purified fragment was found to migrate as a diffuse band on $NaDodSO_4$–polyacrylamide disc gels, devoid of any observable PAS-staining material (Figure 6B). The amino acid composition of the ~55 000-dalton fragment was essentially identical with that published by Markowitz & Marchesi (1981) for the same fragment isolated in $NaDodSO_4$ by a different procedure. Like band 3 in situ, treatment of the reconstituted, membrane-spanning fragment of band 3 with cold, 0.01 N NaOH exerted relatively little affect on the fragment's calorimetric endotherm (not shown). However, exposure of the reconstituted domain to 0.17 M acetic acid resulted in complete loss of the domain's endotherm (compare Figures 5 and 6A). Conversely, the isolated cytoplasmic domain of band 3 was clearly perturbed by cold 0.01 N NaOH (Figure 6A) but essentially unaltered by 0.17 M acetic acid (not shown). These similar sensitivities of intact band 3 and its isolated component domains to two common stripping procedures further support

[5] The reduced temperature of the reconstituted C transition (~72 °C) may derive from an abnormal lipid environment, since addition of 25 mol % bovine brain phosphatidylserine elevates the reconstituted transition to 75 °C (Davio & Low, 1982).

the hypothesis that the structural properties of the joined and cleaved domains of band 3 are not significantly different.

Discussion

The degree of interaction among the component domains of a multidomain protein may vary considerably (Wetlaufer, 1973; Irace et al., 1981). In some cases, the interdomain interactions are so extensive that the protein behaves as a single, uniformly folded polypeptide. The presence of more than one domain in this class of proteins is generally revealed only after careful analysis of the three-dimensional structure of the polypeptide (Wetlaufer, 1973; Levine et al., 1978). In other cases, the association between domains is weak, and the structural transitions within the domains can occur somewhat independently of each other (Ploplis et al., 1981; Donovan & Mihalyi, 1974; Williams & Swenson, 1981). When this situation prevails, the influence of one domain on the structural transitions/behavior of the other(s) can be used as a rough measure of the degree of interaction among the various domains. In accordance with this principle, we have attempted to assess the structural interdependence of the integral and cytoplasmic domains of band 3 by determining (1) whether the integral and cytoplasmic domains of band 3 behave differently after separation by proteolytic cleavage and (2) whether the two regions of the protein unfold independently of each other, i.e., whether one domain senses the structural condition of the other.

The answer to the first of these two questions is peculiarly different for the two domains of band 3. Removal of the cytoplasmic domain exerted no significant effect on the thermal stability of the membrane-spanning region of the polypeptide (Figure 1B). Furthermore, the reconstituted membrane-spanning domain responded to the various membrane stripping procedures in a similar manner to intact band 3 (Figures 5 and 6). Thus, within the resolution of the calorimetric technique, the membrane-spanning domain does not behave differently when disjoined from the cytoplasmic pole of the protein. On the other hand, a small but distinct difference was observed between the thermal stability of the cytoplasmic domain in situ and its stability in isolated form (Figure 2). The lower transition temperature of the membane-associated domain indicates that the region is forced into a slightly unfavorable posture in situ and that this strain is releaved upon cleavage. Whether the induced strain derives from a weak interaction with the integral domain of band 3 or from an interaction with other membrane components cannot be determined unequivocally from the data. However, the latter interpretation appears more likely, since a similar stabilization is not observed in the membrane-spanning domain. Thus, if the change in stability of the cytoplasmic domain upon isolation from the membrane is due to a change in the interactive free energy between the two domains, then a similar stabilization of the membrane-spanning domain should also be observed. This does not occur, and thus the increased stability of the isolated cytoplasmic domain would appear to derive from its separation from other membrane components and not from the integral domain. Furthermore, the overall similarities in the calorimetric and fluorescence behavior of the cytoplasmic domain before and after cleavage from the membrane (Figures 2 and 3), and the similarity in the sensitivity to NaOH and acetic acid stripping (Figures 5 and 6), also suggest that any interdomain interactions must be minimal.

Analysis of the second of the above two questions also supports the contention that the two major domains of band 3 are structurally independent. Thus, the thermal stability of the cytoplasmic domain was not influenced by the structural integrity of the membrane-spanning domain of band 3; i.e., regardless of whether the integral domain was intact or had already disrupted, the temperature of the B_2 transition remained unchanged (Figure 4). Further, either of the two major domains of band 3 could be chemically denatured without significantly perturbing the other domain (Figure 5). Clearly, neither major domain of band 3 is strongly dependent on the complementary domain for stability.

In view of this evidence for little or no interdomain interaction, the seemingly contradictory data of Salhany et al. (1980) and Rice & Steck (1977) require some comment. Salhany and co-workers reported that DIDS binding to the integral domain of band 3 modifies hemoglobin binding to the cytoplasmic domain. While this behavior clearly implies some interdomain communication, this communication need not be mediated through a direct interdomain contact. Thus, DIDS labeling is known to exert profound effects on erythrocyte membrane architecture (Snow et al., 1978; Singer & Morrison, 1980; Eaton et al., 1980). Since the cytoplasmic domain of band 3 is thought to be surrounded by numerous other components of the membrane (Lux, 1979), a general membrane perturbation might be expected to alter the accessibility of the cytoplasmic domain to potential protein ligands, e.g., hemoglobin. Alternatively, the DIDS modification of hemoglobin binding might derive from an electrostatic perturbation of the band 3 environment. The binding of two DIDS molecules to a band 3 dimer should remove four cationic groups, i.e., basic amino acid side chains, and introduce four anionic (sulfonate) groups into the same region of the dimer (Jennings & Passow, 1979). This net change of eight electrostatic charges could influence the disposition of the cytoplasmic domain against the membrane. If the hemoglobin site changes orientation/environment during such a rearrangement, an altered hemoglobin binding isotherm might be expected.

The partial inhibition of pyruvate transport by disulfide cross-linking of associated cytoplasmic domains (Rice & Steck, 1977) also may not be inconsistent with our observations.[6] If the steric or electrostatic environment of the entrance to the anion channel is determined in part by the orientation of the attached cytoplasmic domain, then any reorganization of this region of the transport protein might be expected to influence the properties of anion transport. Unfortunately, no such rearrangement of the cytoplasmic domain has been demonstrated, and we observe no effect of disulfide cross-linking on the calorimetry of the cytoplasmic domain. Thus, whether the observations of Steck, Salhany, and their co-workers (Salhany et al., 1980; Rice & Steck, 1977) imply an interdomain contact too weak to be detected by calorimetry or whether their observations can be correctly explained by alternative mechanisms must await further information.

The calorimetric data on the variously stripped erythrocyte membranes may be useful in predicting the functional properties of the resulting IOV's. Assuming that a denatured domain will not function correctly, one might predict that the cytoplasmic domain will bind ankyrin in KCl- and acetic acid stripped membranes but with reduced efficiency in NaOH-stripped membranes. The data of Hargreaves et al. (1980) and Bennett & Stenbuck (1980) clearly bear this out. Thus, Hargreaves and co-workers found that ankyrin binding to the cytoplasmic domain of band 3 was effective in KCl-stripped membranes but significantly reduced in NaOH-stripped membranes. Bennett & Stenbuck (1980) have also shown that

[6] We have assumed that pyruvate transport is mediated by band 3. If this is not the case, then no apparent contradiction exists.

the isolated cytoplasmic fragment derived from acetic acid stripped vesicles still retains the ability to bind ankyrin. This same line of reasoning should also pertain to the integral domain of band 3. In this case, vesicles derived from either KCl- or NaOH-stripped membranes should transport anions relatively normally, but anion exchange should be greatly inhibited across acetic acid stripped membranes. Wolosin et al. (1977) have shown that membranes incubated in the presence of NaOH to remove all peripheral proteins retain the ability to transport anions. However, no data on KCl- or acetic acid stripped membranes have been reported.

Finally, the identification of the B_2 endotherm as the unfolding transition of the cytoplasmic domain of band 3 allows the interpretation of some previous calorimetric data. In an earlier study of the effect of adenine nucleotides on the behavior of spectrin in situ, it was observed that low concentrations of ADP displace the B_2 transition (Low & Brandts, 1978). In other published (Appell & Low, 1981; Brandts et al., 1978; Snow et al., 1978) and unpublished studies, the B_2 transition has been shown to be sensitive to phloretin, Ca^{2+}, ionic strength, pH, and 2,3-diphosphoglycerate. These perturbations indicate that the stability of the cytoplasmic domain of band 3 is modified by the presence of these ligands/solution conditions. The most reasonable explanation of this behavior is that specific sites exist on band 3 for each of the above perturbants and that ligand binding alters the structure/stability of the cytoplasmic domain. These possibilities are currently under investigation.

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