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Protein Involvement in Structural Transitions of Erythrocyte Ghosts. Use of Thermal Gel Analysis To Detect Protein Aggregation†

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ABSTRACT: In this study, it is shown that systematic temperature-induced protein aggregation occurs on the erythrocyte membrane by intermolecular disulfide bond formation. Specific protein bands disappear from acrylamide gel profiles over rather narrow temperature regions. The aggregation appears to be the result of irreversible structural transitions of the membrane, which can be seen in a sensitive scanning calorimeter. When this method of thermal gel analysis is used, the results suggest that spectrin is a participant in the A transition, that bands 2.1, 4.1, and 4.2 and the cytoplasm portion of 3 are involved in the B transition, and that the transmembrane portion of band 3 may undergo changes in the C transition, previously shown to occur in the anion transport domain of the membrane. The aggregation of specific proteins in the narrow temperature region of these transitions persists as the transitions are moved around on the temperature axis by varying solution conditions. The assignment of particular proteins to specific transitions is reinforced by selective extraction of membrane proteins. Large variations in both the calorimetry and the aggregation pattern occur as salt concentration is increased from 77 mosm to 310 mosm, which is manifested in the splitting of the B transition into two separate transitions, B_1 and B_2. It is speculated that this occurs as the result of a structural change which may involve components of the cytoskeletal network.

When an ultrasensitive scanning calorimeter is used, five distinct thermal transitions can be seen to occur in human erythrocyte membranes over the temperature region 45-80 °C (Jackson et al., 1973; Brandts et al., 1978). Each of these transitions presumably occurs as the result of "melting" or disorganization of a cooperative domain on the membrane. One of these transitions (the A transition) was previously shown (Brandts et al., 1977) to be due to the partial unfolding of the spectrin complex and results in the loss of the characteristic biconcave shape of the ghosts. Another (the C transition) is now known to occur in the membrane domain which

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functions in anion transport (Snow et al., 1978, 1981).

Calorimetry in itself is not extremely useful in identifying particular membrane components that are involved in structural transitions. Consequently, a new method of characterization, call thermal gel analysis (TGA), has been developed in our laboratory, and the application of this technique to the transitions of erythrocyte ghosts gives further information on the participation of specific proteins in these transitions.

The rationale for the TGA method is straightforward. Protein participation in cooperative transitions may involve protein denaturation and/or changes in the pattern of protein–protein associations on the membrane. Denaturation might also lead indirectly to changes in the state of aggregation as it is known to do for many soluble proteins. Near neutral pH, it can further be expected that intermolecular disulfide bond formation may produce covalent cross-links between proteins which are in close proximity on the membrane, particularly if one or more of the proteins have been partially unfolded so that potential cross-linking sites are available for reaction.

Since all of the erythrocyte transitions are irreversible, cross-links that form during thermal transitions should be evident upon running solubilized membrane samples on polyacrylamide gels if no dithiothreitol or other sulfhydryl reagent is present to destroy the cross-links. Thus, protein association will be visualized by the disappearance of specific protein bands and the appearance of high molecular weight aggregates on the gel. If the formation of disulfide cross-links results directly from structural changes that occur in a specific membrane transition, then the examination of numerous samples that have been heated to different maximal temperatures should show a correlation with the calorimetric transition. That is, the temperature which corresponds to the loss of 50% of the gel area ($T_{1/2}$) for a particular protein band should be identical with the midpoint (T_M) of the calorimetric transition if that protein is actually a participant in the transition. It is always possible, however, that the aggregation temperature for a protein might accidentally coincide with the T_M for a structural transition. To eliminate this possibility, it is desirable to shift the T_M for the structural transition by varying conditions and seeing if this produces a corresponding shift in the aggregation temperature, $T_{1/2}$.

As will be seen, our results show that certain transitions involve the simultaneous aggregation of many proteins, while other transitions seem not to be associated with any protein aggregation that can be visualized by TGA. The involvement of a number of different proteins in a single transition implies that all of these proteins are participants in the same cooperative structural domain of the membrane. Thus, information on the pattern of protein–protein interactions on the native membrane can be obtained from TGA. Using this logic, we are led to the conclusion that an extensive interactive network exists, involving bands 2.1, 3, 4.1, and 4.2 and perhaps spectrin (i.e., bands 1 and 2) as well.

Experimental Procedures

Calorimetric Studies. Most of these studies were carried out with a home-built instrument described previously (Jackson & Brandts, 1970), using 1-mL matched platinum cells. Some of the more recent data were obtained on a more sensitive commercial scanning calorimeter (MicroCal Inc., Amherst, MA). The scanning rates were either 40 or 60 °C/h, with no detectable differences due to scan rate. Membrane concentrations were close to 1% by weight, unless otherwise noted. Noise levels in the traces were only ~2% as large as deflections due to the larger transitions.

$NaDodSO_4$[1]–Polyacrylamide Gel Electrophoresis. Membranes were solubilized in a solubilizing buffer (0.02 M Tris, 0.002 M EDTA, pH 8.0, and 0.2% $NaDodSO_4$, with or without 2% dithiothreitol). The gels were 5.6% acrylamide with 4% cross-linking. After running, the gels were placed overnight in a fixing solution (10% trichloroacetic acid and 20% methanol in distilled water). They were stained with 0.25% Coomassie blue in a 5:1:5 (v/v) ratio of water/acetic acid/methanol solution for ~1.5 h. Destaining was carried out in a 7% acetic acid solution. The gels were scanned on a Gilford spectrophotometer. The use of PAS staining followed the procedure of Zacharius et al. (1969).

Thermal Gel Analysis Technique. For a thermal gel study, individual aliquots (50–100 μL) of membrane sample were placed in small corked test tubes. The tubes were put into a water bath which was heated at a rate close to that of the calorimeter. The test tubes were removed from the bath at various temperatures and cooled to room temperature. After all of the samples were removed, they were adjusted to 77.5 mosm phosphate buffer, pH 7.4, by the addition of the appropriate solution. The samples were then stored at 4 °C until they were solubilized. Standardization of the incubation conditions enabled us to be certain that changes in the aggregation pattern were the result of the heating conditions and not of the incubation conditions.

After incubation, the samples were solubilized (1:1 v/v) by the addition of solubilization buffer without dithiothreitol. The samples were electrophoresed on $NaDodSO_4$–acrylamide gels.

After the stained gels were scanned, the peak areas for each band were determined with a planimeter, and the area was normalized with respect to the 25 °C sample. This normalized area was then plotted vs. temperature to obtain the curves from which the thermal midpoint ($T_{1/2}$) was determined.

Results

Irreversible Protein Aggregation. When a suspension of erythrocyte ghosts in 310 mosm phosphate buffer, pH 7.4, is heated to 75 °C and then solubilized in $NaDodSO_4$ buffer in the absence of dithiothreitol, the acrylamide gel pattern changes markedly. All of the Coomassie blue staining protein bands are essentially absent in the gel scan. A high molecular weight "plug" is evident at the top of the gel. [It was ascertained in separate control experiments that the presence of the plug itself does not prevent nonaggregated protein (e.g., serum albumin) from entering the gel.] Presumably, the loss of protein bands is due to irreversible aggregation resulting from the exposure to high temperature.

The nature of the interactions which stabilize the aggregates was determined experimentally. The addition of 8 M urea to the solubilization buffer does not change the gel pattern to any appreciable extent. This suggests that noncovalent interactions cannot be solely responsible for the aggregates since 8 M urea should break these. However, if 0.8 M dithiothreitol is added to the incubation buffer instead of urea, then the normal protein banding pattern reappears in the gel. The disaggregation of proteins caused by the presence of dithiothreitol is fairly complete with the exception of the spectrin components (bands 1 and 2). Although these reappear to a large extent, their concentration on the gel is lower than expected if disaggregation were complete.

Thus, the heating of erythrocyte membranes cause extensive aggregation of the membrane proteins, and reversal of the aggregation process by dithiothreitol shows that intermolecular

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; TGA, thermal gel analysis.

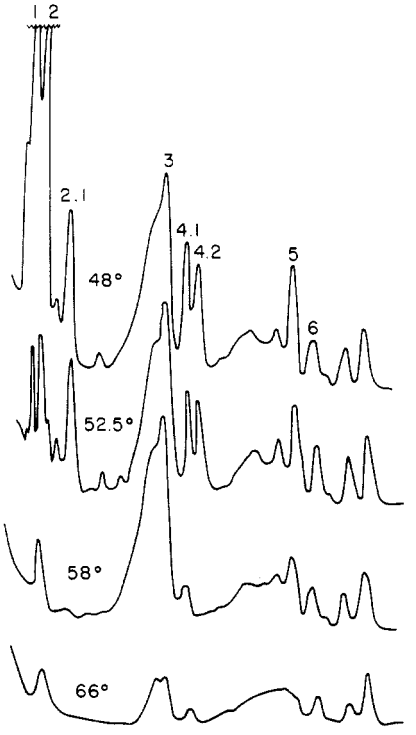

FIGURE 1: Gel scans on aliquots of erythrocyte membranes heated to different maximal temperatures. The first scan (48 °C) is prior to the occurrence of any transitions, the second (52.5 °C) is just after the A transition, the third (58 °C) is subsequent to the B_1 transition, and the last (66 °C) is after the B_2 transition.

disulfide bonding plays a role in the aggregation process.

Kinetic studies indicate that the formation of aggregates occurs quickly. Samples which were solubilized immediately after a given temperature was reached have nearly the same extent of aggregation as found for samples which were subsequently incubated (4 °C) for up to 48 h before being solubilized.

Temperature Dependence of Aggregation at pH 7.4. The disappearance from gels of the various protein bands does not occur uniformly with exposure to increasing temperatures. Rather, protein bands disappear cooperatively over narrow temperature regions, and the specific temperature region is different for different proteins. Shown in Figure 1 are gel scans taken on identical samples which had been heated (60 °C/h) to different maximal temperatures in a high salt buffer (310 mosm phosphate, pH 7.4) prior to being solubilized in the absence of dithiothreitol and then electrophoresed. The gel pattern remains unchanged up to temperatures of ~48 °C. Between 48 and 52.5 °C, the spectrin components (bands 1 and 2) aggregate and disappear from the gel. Further heating to 58 °C causes the additional loss of bands 2.1, 4.1, and 4.2, as seen in Figure 1. Note that the intensity of band 3 has scarcely been affected by heating to 58 °C but that it disappears very cooperatively with further increase in temperature to 66 °C. Those proteins with apparent molecular weights smaller than band 4.2 also disappear with increasing temperature, but in a more gradual and less reproducible manner than for the larger proteins.

The relative areas for each of the high molecular weight proteins (bands 1, 2, 2.1, 3, 4.1, and 4.2), as a function of the maximum temperature to which the samples were heated, are shown in Figure 2. These data can be compared to the calorimetric scan obtained on an aliquot of the same solution using the same heating rate (Figure 3, trace 1). It can be seen that the midpoint for the disappearance ($T_{1/2}$) of bands 1 and 2 is 50 °C, or nearly coincident with the calorimetric A

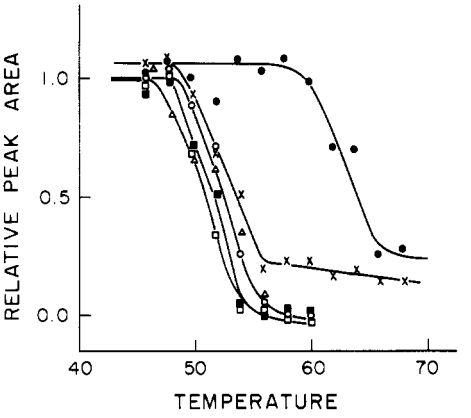

FIGURE 2: Normalized relative peak areas for major membrane proteins, plotted as a function of the maximum temperature to which the membranes were heated in a 310 mosm phosphate buffer, pH 7.4. Included are areas for bands 1 (□), 2 (■), 2.1 (△), 3 (●), 4.1 (×), and 4.2 (○).

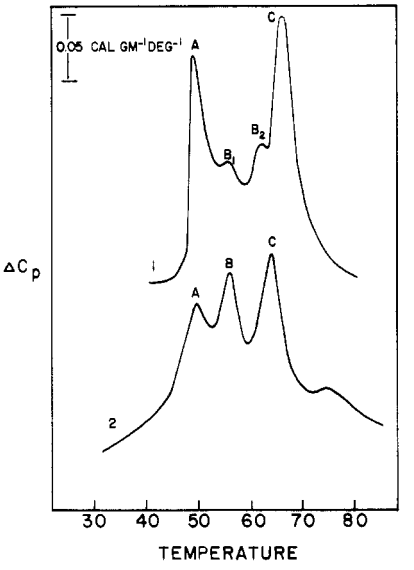

FIGURE 3: Calorimetric scans for membranes in suspending buffers of 310 mosm phosphate, pH 7.4 (trace 1), and in 775 mosm phosphate, pH 7.4 (trace 2).

transition. The disappearance of bands 2.1, 4.1, and 4.2 from the gels occurs nearly simultaneously, each having a $T_{1/2}$ of 53 ± 1 °C. This is very close to the midpoint of the calorimetric B_1 transition under these conditions (53.5 °C). The last of the high molecular weight proteins to aggregate is band 3 with a $T_{1/2}$ of 62 °C, nearly coincident with the calorimetric B_2 transition (61 °C).

Because of the cooperativity with which the protein bands disappear from the gels, it seems possible that their disappearance is actually the result of the same structural changes which are responsible for the calorimetric transitions that occur at the same temperatures. For a more careful exploration of this hypothesis, an experiment very similar to that shown in Figure 2 was carried out, but in a less concentrated phosphate buffer containing only 77 mosm sodium phosphate. It was shown earlier that as buffer concentration is reduced, the B_1 transition shifts to higher temperature and the B_2 transition shifts to lower temperature, so that at 77 mosm the two transitions have merged into a single B transition located at an intermediate temperature (Brandts et al., 1978), as shown calorimetrically in Figure 3 (trace 2). The thermal gel patterns are also drasticaly modified under these low salt conditions (Figure 4), with the exception of bands 1 and 2, which still show a $T_{1/2}$ of 49 °C. However, the gel transitions of bands

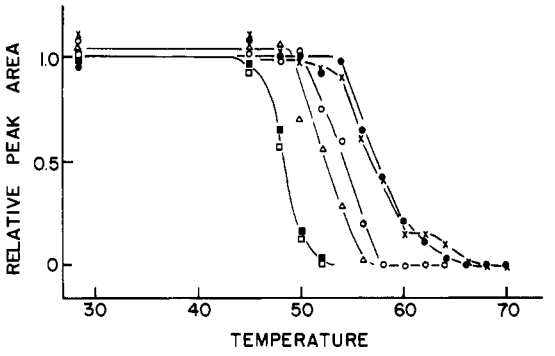

FIGURE 4: Normalized relative peak areas for major membrane proteins, plotted as a function of the maximum temperature to which the membranes were heated in a 77 mosm phosphate buffer, pH 7.4. Included are areas for bands 1 (□), 2 (■), 2.1 (△), 3 (●), 4.1 (×), and 4.2 (○).

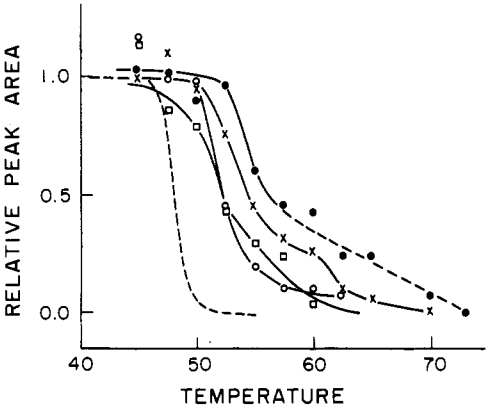

FIGURE 5: Results of thermal gels for membranes suspended in 310 mosm phosphate buffer, pH 8.15. Included are relative areas for band 1 and 2 (---), 2.1 (□), 3 (●), 4.1 (×), and 4.2 (○).

2.1, 4.1, and 4.2 all shift to higher temperature, while the transition of band 3 shifts to lower temperature, so that all four of these proteins show a $T_{1/2}$ of 56 ± 2.5 °C. Under these conditions, the coupled B transition has a calorimetric T_m of 56 °C.

These results using the thermal gel method suggest that bands 1 and 2 are involved in the calorimetric A transition, since $T_{1/2}$ closely correlates with T_m. This agrees with earlier work where it was shown that the spectrin complex unfolds in the A transition (Brandts et al., 1977). The thermal gel results are also consistent with the idea that bands 3, 2.1, 4.1, and 4.2 may be involved in the calorimetric B transition. The splitting of the B transition which occurs in high salt buffers is accompanied by a separation in the thermal gel "transitions" of these four bands, suggestting that band 3 undergoes aggregation in the B_2 transition, while bands 2.1, 4.1, and 4.2 are similarly affected in the B_1 transition. The glycoproteins were examined in separate experiments and were found not to aggregate significantly anywhere up to 75 °C.

Results at pH 6.35 and 8.15. In 310 mosm phosphate buffer, the calorimetric B_2 transition is very pH dependent while the A, B_1, and C transitions show very little pH dependence of their T_M's (Brandts et al., 1978). The midpoint for the B_2 transitions shifts from about 70 °C at pH 6 to 52 °C at pH 9, exhibiting an apparent pK of 7.5. If the aggregation of band 3 is truly associated with the B_2 transition, then the "transition" of band 3 seen by TGA should also show this strong pH dependence.

As can be seen in Figure 5, the transitions delineated by the thermal gel experiments at pH 8.15 are much different than those at pH 7.4. In this case, the midpoint of the transitions

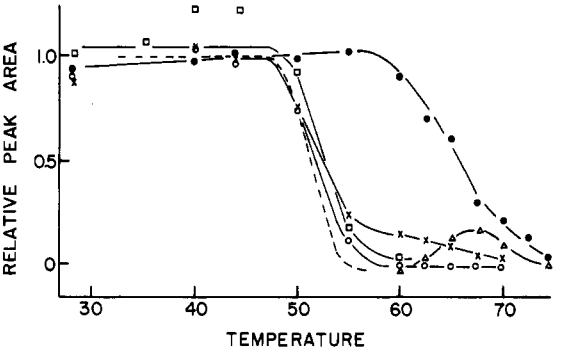

FIGURE 6: Results of thermal gels for membranes suspended in 310 mosm phosphate buffer, pH 6.35. Included are relative areas for bands 1 and 2 (---), 2.1 (□), 3 (●), 4.1 (×), and 4.2 (○). The triangles (△) show the appearance and disappearance of a new band of M_r 220 000.

for bands 2.1, 4.1, and 4.2 are at about 53 °C. This value of $T_{1/2}$ is very close to T_M for the calorimetric B_1 transition which is at 52.5 °C under these conditions. For band 3 the $T_{1/2}$ is close to 55 °C while the calorimetric B_2 transition is at 54 °C. As was seen earlier at pH 7.4 (Figure 4), it also appears here that some of band 3 does not disappear in the 55 °C transition but seems to go away at a higher temperature near the calorimetric C transition (67 °C). Bands 1 and 2 (data not shown) disappear from the gel with a midpoint of about 48 °C, which is exactly coincident with the calorimetric midpoint of the A transition at this pH.

At pH 6.35, the gel transition for bands 1 and 2 is at 50 °C, which is again coincident with the calorimetric A transition at the same pH (49.5 °C). Shown in Figure 6 are the data for bands 2.1, 4.1, and 4.2, whose transitions occur virtually simultaneously with a $T_{1/2}$ of 52 °C, i.e., very near the calorimetric B_1 transition (53 °C). The largest difference between the pH 6.35 and 8.15 data is for the band 3 transition which at pH 6.35 occurs with a $T_{1/2}$ of about 67 °C as compared with a T_M of 70 °C for the calorimetric B_2 transition. Thus, the shift in the calorimetric B_2 transition in going from basic to acid pH is accompanied by a nearly corresponding shift in the gel transition for band 3.

One final point is worth emphasis. At pH 6.35, a new band begins to appear in the gel scan at a temperature of ~65 °C, reaches a maximum intensity, and then disappears again near 75 °C, as shown in Figure 6. This migrates in the appropriate position to be a dimer of band 3. Although this band can be seen in most of our TGA experiments, its area is largest when there is a moderately large temperature separation between the B_2 and C transitions.

Effect of Dimethyl Sulfoxide Addition. The progressive addition of dimethyl sulfoxide Me_2SO changes the calorimetric behavior of ghosts. At low Me_2SO concentrations, the B_1 and B_2 transitions fuse together when the suspending buffer is 310 mosm phosphate, pH 7.4 (unpublished observations). As higher concentrations are added, this coupled B transition as well as the A transition is shifted to progressively lower temperatures. In addition to the shifts in the thermal midpoints of the A and the coupled B transitions, there is a reduction in the size of the A transition and an increase in the size of the B transition in dilute Me_2SO. The C transition is not as strongly affected by Me_2SO.

We have carried out TGA studies at a concentration of 2.5 M Me_2SO. Under these conditions, the large coupled B transition occurs with a midpoint near 50 °C while the A transition has shifted down to 43 °C. The C transition occurs at 63.5°, which is only 2.5 °C lower than in the absence of Me_2SO. The TGA transitions of bands 1 and 2 (data not

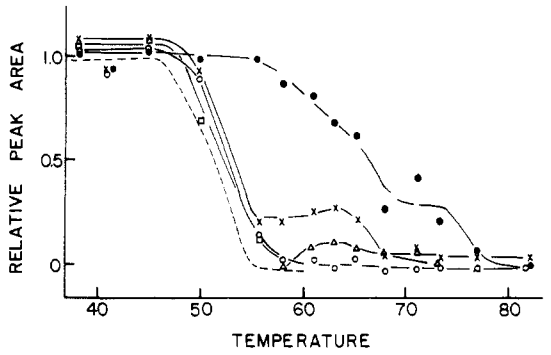

FIGURE 7: Results of thermal gels for SITS-treated membrane in 310 mosm phosphate buffer, pH 7.4. Symbols are identical with those of Figure 6.

shown) occur with the midpoint near 44 °C, very close to T_M for the A transition. Band 2.1 has a $T_{1/2}$ of ca. ~46.5°, while bands 4.1 and 4.2 have a midpoint of about 48.5 °C. The band 3 transition occurs at 49.5 °C, although, as before, a small amount of band 3 appears not to participate in the main transition. These results show that the presence of 2.5 M Me_2SO shifts the calorimetric A transition and the thermal gel transition of bands 1 and 2 by a comparable amount. It also recouples the calorimetric B_1 and B_2 transitions and shifts them down by a substantial amount while at the same time causing the thermal gel transitions of band 3 to nearly "rejoin" the thermal gel transitions of bands 2.1, 4.1, and 4.2 at the reduced temperature. This is an important point since under these identical conditions, but in the absence of Me_2SO, the $T_{1/2}$ of band 3 differs from that of bands 4.1, 4.2, and 2.1 by about 10 °C (cf. Figure 2).

Results on SITS-Trated Membranes. Membranes which lack the ability to undergo the C transition are also seriously impaired in their ability to transport anions (Snow et al., 1981). Also a number of specific covalent inhibitors of anion transport shift the C transition to higher temperatures by as much as 13 °C without affecting any other transitions (Snow et al., 1978). Since band 3 is the principal protein involved in anion transport (Steck, 1978; Rothstein et al., 1979), this protein may also be involved in the C transition, and some of our earlier experiments show indications of this. In order to examine this possibility more closely, we have carried out TGA studies on SITS-treated membranes, where the C (T_M = 78 °C) and B_2 transitions (T_M = 62 °C) are well separated on the temperature axis.

The TGA data (Figure 7) show a sharp transition for bands 1 and 2 with a midpoint of 50 °C, which is identical with the T_M for the A transition. Bands 2.1, 4.1, and 4.2 all have a $T_{1/2}$ near 53 °C, while the T_M for B_1 is 55.5 °C under these conditions. The major portion of band 3 disappears with a $T_{1/2}$ of ~63 °C, very close to the B_2 transition, but a smaller amount (~25%) does not disappear until a temperature close to the C transition. Another interesting phenomenon which becomes more apparent when C is shifted to 78 °C is that as band 3 begins to aggregate near 58 °C, a new band, mentioned earlier, appears at M_r ~200 000 and then disappears again at higher temperatures where band 3 becomes totally aggregated. This new band is likely a dimer of band 3.

Results on DMMA-Extracted Membranes. The TGA results discussed above suggest that bands 1 and 2 are involved in the A transition, bands 4.1, 4.2, and 2.1 in the B_1 transition, and band 3 in the B_2 transition (and possibly also in the C transition). As previously found, membranes from which only bands 1, 2, and 5 have been extracted lack the A transition (Brandts et al., 1977). These membranes display a normal

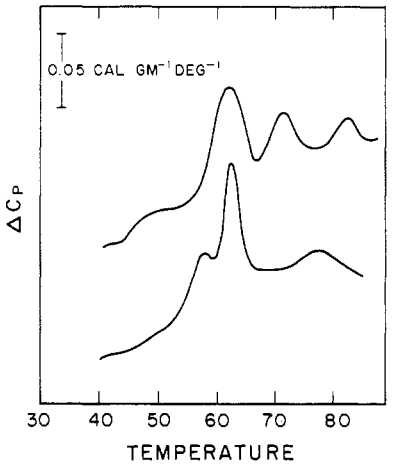

FIGURE 8: Calorimetric scans on residual membranes resulting from DMMA extraction. The suspending buffer was 77 mosm phosphate, pH 7.4 (lower trace), and 310 mosm phosphate, pH 6.15 (upper trace).

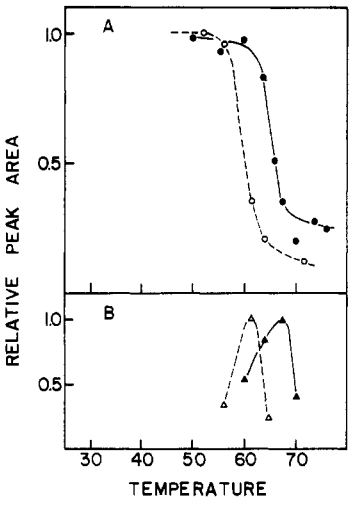

FIGURE 9: Thermal gel results on DMMA-extracted membranes in 77 mosm phosphate, pH 7.4 (---), and in 310 mosm phosphate, pH 6.15 (—). (A) Relative areas for band 3. (B) Relative areas for aggregation product of M_r 220 000.

B, C, and D transition in 77 mosm phosphate buffer, pH 7.4. Extraction of ghosts with dimethylmaleic anhydride (DMMA) results in the solubilization of bands 2.1, 4.1, 4.2, and 6 in addition to bands 1, 2, and 5 (Steck & Yu, 1973; Kahlenberg, 1976). Our gel scans of membranes which have been extracted with DMMA show nearly complete extraction of all proteins except bands 3 and 7 and a broad band (4.5) of intermediate molecular weight. Also, all of the PAS-staining bands remain on the membrane.

Calorimetric scans of DMMA-extracted ghosts are shown in Figure 8 at pH values of 7.4 and 6.15. These membranes lack both the A and B_1 transition. The B_2, C, and D transitions occur at their normal temperatures, unchanged by the extraction of about 50% of the membrane protein. The large pH dependence of T_M for the B_2 transition is still apparent in the comparison of the two scans at pH 7.4 and 6.15.

The results of TGA studies are seen in Figure 9 at both pH values. It can be seen that the $T_{1/2}$ for aggregation of band 3 does shift substantially higher (by ~7 °C) in going from pH 7.4 to 6.15, but this is somewhat less than the shift in calorimetric T_M for the B_2 transition (~11 °C) seen in Figure 8. This is probably due to the aforementioned tendency for a small amount of band 3 to aggregate in the C transition, which occurs below B_2 at pH 6.15 but above B_2 at pH 7.4.

The new band of M_r ~200 000 which forms at high tem-

perature is very clearly seen in these DMMA-extracted membranes. Its area is maximal at 60 °C in the pH 7.4 buffer and at 68 °C in the pH 6.15 buffer, as seen in Figure 9. Since all other proteins which might contribute to the formation of this aggregate have been removed by extraction, it appears certain that this must be a dimer of band 3.

These results confirm the fact that bands 2.1, 4.1, and 4.2 are involved in the B_1 transition. This follows rather directly since extraction of bands 1, 2, and 5 by low salt exposure leads to loss of the A transition, while the additional extraction of bands 2.1, 4.1, 4.2, and 6 by DMMA leads to loss of B_1, in addition to A. Since band 6 can be extracted (high salt) without the loss of any transitions, it appears that the disappearance of B_1 must be due to removal of any or all of bands 2.1, 4.1, and 4.2, which agrees nicely with TGA results.

The Aggregation of Band 3′. Band 3′ is a very sharp band on the leading edge of band 3. It has been suggested that this is a proteolysis product of band 2.1 (Luna et al., 1979). It is not known whether band 3′ occurs on the membrane in vivo or whether it is formed during preparation of ghosts. Its concentration in ghosts is quite variable.

In several TGA experiments, band 3′ could be separately resolved from band 3. Although quantitative areas are difficult to determine for band 3′ on acrylamide gels, a good qualitative indicator of its aggregation behavior could be obtained. Band 3′ clearly disappears from the gels almost coincident with band 2.1 in experiments where it could be seen clearly. Thus, band 3′ is indistinguishable from 2.1 in terms of its aggregation behavior and clearly different from band 3, consistent with the idea that it is a proteolysis product of 2.1.

Discussion

Thermal gel analysis is able to detect systematic protein aggregation that occurs during thermally induced structural transitions. Examination of the temperature dependence of band areas shows that bands 1 and 2 aggregate cooperatively over a narrow temperature region which coincides with the T_M of the A transition in all buffers tested. Spectrin is known to lose 30% of its apparent helical content in the A transition (Brandts et al., 1977), which leads to sphering of the ghosts and presumably facilitates aggregation as well.

In buffers of low salt concentration (77 mosm), bands 2.1, 4.1, 4.2, and 3 (and probably 3′ as well) aggregate at temperatures very close to the B transition. At higher salt concentrations (310 mosm), band 3 alone aggregates in the B_2 transition, while bands 2.1, 4.1, and 4.2 aggregate in the B_1 transition. This "assignment" of specific proteins to calorimetric transitions has been confirmed in two ways. First, when the transitions are shifted along the temperature axis by varying pH or salt concentrations or by adding potent effectors such as SITS or dioxane, it is observed that the aggregation temperatures shift in a predictable way. Second, specific extraction of bands 1 and 2 has been shown to cause the loss of the A transition, while the additional extraction of 2.1, 4.1, and 4.2 leads to the loss of the B_1 transition as well.

The simultaneous aggregation of a number of proteins during a single cooperative transition may be a manifestation of their close physical proximity in a membrane domain or of their coupling through an interacting network. There are numerous observations in the literature which indicate that the proteins which coaggregate in the B transition do, in fact, interact with one another, either directly or indirectly. Band 4.2, for example, is eluted in as a 1:1 complex with band 3 during nonionic detergent extraction (Steck, 1978). Bennett & Stenbuck (1979a,b) have shown strong interaction between band 2.1 and the complex of bands 4.2 and 3. Band 2.1 is also thought to contain one of the sites at which spectrin attaches to the membrane (Tyler et al., 1979). It has been proposed (Luna et al., 1979) that band 3′ is a proteolytic fragment of band 2.1; so this also may interact with band 3. Although there is no direct evidence linking band 4.1 directly to the other proteins involved in the B transition, it does link at least indirectly to band 2.1 through their mutual interaction with spectrin (Tyler et al., 1979).

These results using TGA are then consistent with a membrane structure which involves interactions between bands 2.1, 4.1, and 4.2 and band 3 at low salt. Other studies utilizing fluorescent sulfhydryl reagents (unpublished results) suggest a further interaction of bands 1 and 2 with any or all of bands 2.1, 4.1, and 4.2 since labeling of these latter proteins causes a large decrease in area for the A transition, even though spectrin itself is not labeled. All six of these proteins have been identified as direct or indirect participants in the cytoskeleton structure of the erythrocyte membrane (Lux, 1979; Tyler et al., 1979; Bennett & Stenbuck, 1979a,b; Sheetz, 1979).

At high salt, band 3 no longer aggregates together with bands 4.1, 4.2, and 2.1 in the B_1 transition but remains monomeric until temperatures which coincide with the onset of the B_2 transition. Evidently, there is some reorganization of the membrane as the salt concentration is increased at pH 7.4. Earlier work (Brandts et al., 1978) has shown that the splitting into B_1 and B_2 is centered near a salt concentration of $\sim$200 mosm at pH 7.4. Yu & Steck (1975) have shown that band 6 (glyceraldehyde-3-phosphate dehydrogenase), which binds to band 3 at low salt, is dissociated from the membrane in a moderately cooperative process also centered close to 200 mosm at pH 7.4. A second glycolytic enzyme, aldolase, has also been shown to bind to band 3 at low salt (Strapazon & Steck, 1977) and to dissociate under nearly the same conditions noted above. Since splitting of the B transition also occurs for membranes from which band 6 has been removed (unpublished observations), it appears likely that the dissociation of these enzymes is not the cause of the splitting but that both are the result of a structural reorganization of the membrane. This idea agrees with the results of Jenkins & Tanner (1977), who interpreted salt-dependent changes in the proteolytic susceptibility of band 3 as being due to a membrane reorganization which leads to a "tighter" structure for band 3 at higher salt concentrations. This salt-sensitive structural reorganization of these cytoskeletal proteins, accompanied by the release of two glycolytic enzymes from the membrane, may have some physiological significance.

A small portion of band 3 disappears from the gels in the C transition under conditions where the B_2 and C transition are well separated on the temperature axis. It is not surprising that band 3 might also be involved in the C transition in view of the high sensitivity of this transition to the binding of anion transport inhibitors to band 3. We feel that the involvement of band 3 in two separate transitions may be a reflection of the subunit-like structure of this protein and its organization into relatively independent domains. This is also suggested from proteolysis studies (Snow et al., 1981), where it was shown that external proteolysis of band 3 with chymotrypsin has no effect on any of the transitions, while extensive cleavage from the cytoplasm side causes loss of the B_2 transition. The C transition is insensitive to all types of proteolysis with chymotrypsin, and only the 17K transmembrane fragment of band 3 is necessary for the C transition to occur normally. It seems then that the B_2 transition is associated with the cytoplasm domain of band 3 while the C transition is associated with the transmembrane domain which includes the anion

transport site. Work in another laboratory (P. Low, personal communication) has shown that the B_2 and C transitions can be reconstituted into model systems by using appropriate fragments of band 3 obtained by mild proteolysis and chromatographic separation.

The TGA method is, therefore, capable of providing detailed information on the participation of erythrocyte proteins in various cooperative structural changes occurring in the membrane. The method depends on thermal cross-linking through the formation of intermolecular disulfide bonds, which are in all likelihood triggered by the reorganization of membane components and/or the unfolding of certain proteins. The method appears to have general applicability, and preliminary results on other membrane systems are encouraging.

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Comparative Study of an Adenosine Triphosphatase Trigger-Fused Lipid Vesicle and Other Vesicle Forms of Dimyristoylphosphatidylcholine†

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ABSTRACT: Several known forms of bilayer vesicles of dimyristoylphosphatidylcholine exhibit the gel to liquid-crystalline phase transition in the temperature range convenient for membrane enzyme reconstitution studies. This warrants a systematic investigation of their physical characteristics and their phase transition behaviors. We have employed electron microscopy, gel chromatography, ^{31}P nuclear magnetic resonance, differential scanning microcalorimetry, and fluorescence spectroscopy to determine several physical parameters of the limiting size microvesicle (260 ± 40 Å), the larger vesicle form (900 ± 100 Å) of Enoch and Strittmatter [Enoch, H. G., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145], the multilamellar vesicle, and, in particular, an ATPase-trigger-fused macrovesicle (950 ± 200 Å). This latter vesicle form was produced by a spontaneous fusion of the complex of the plasma membrane ATPase of *Schizosaccharomyces pombe* and the lipid microvesicles at a low ratio of enzyme to vesicle concentrations, and at a low temperature (around 10 °C). The ATPase-trigger-fused vesicles are unilamellar and have an intact ionic permeation barrier at 30 °C and a gel to liquid-crystalline transition temperature at 24.4 °C with a transition heat of 5.64 kcal/mol. Thus, this vesicle form should be a valuable tool for studying possible proton-pumping activity of this ATPase. In contrast to data found in the literature, which show lack of the pretransition for unilamellar microvesicles, we have observed the pretransition around 15 °C for all the vesicle forms examined. Moreover, the transition widths of unilamellar vesicles are much broader than those of the multilamellar vesicles, suggesting that in the latter system interlayer interactions may contribute to the cooperativity of the transition.

Attempts have been made to prepare lipid vesicle systems that possess desired properties for membrane enzyme reconstitution studies. The relevant features of the vesicle systems include, for example, an easy and complete incorporation of proteins, an intact and well-defined ionic permeation barrier, and, most importantly, the retention or the reactivation of the biological activity of proteins. Another useful feature of the lipid vesicles is the phase transition property, which would allow studies of the effect of lipid conformational states on the biological activity of enzymes. Dimyristoyl-

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