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CALORIMETRIC STUDIES OF THE STRUCTURAL TRANSITIONS OF THE HUMAN ERYTHROCYTE MEMBRANE

STUDIES OF THE B AND C TRANSITIONS

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Summary

Differential scanning calorimetry has been used to study several structural transitions of the human erythrocyte membrane. Earlier studies have shown that one of these transitions (the A transition) is due to the thermal unfolding of spectrin on the membrane. In this paper, it is shown that two of the other transitions (B and C) exhibit a high sensitivity to a local anesthetic, benzyl alcohol. Increasing the ionic strength of the suspending medium results in a splitting of the B transition into two independent transitions (B_1 and B_2). It is found that one of these (B_2) is associated with titrating groups, since the midpoint for the transitions shifts by about 20°C, with an apparent pK near 7.5. Extensive bilateral proteolysis by papain causes a drastic decrease in the size of all transitions except the C transition, which remains unaltered. On the other hand, treatment with phospholipase A_2 largely affects the C transition, causing its disappearance. Because of the lack of sensitivity to proteolysis and the high sensitivity to phospholipase, it appears that the C transition has a large extent of 'lipid involvement'. It might result from the melting of a small fraction of phospholipid which exists in a crystalline state under physiological conditions. Alternatively, the C transition could arise from changes in protein-lipid interactions or from lipid-dependent changes in protein-protein interactions, providing one assumes that only protease-resistant portions of membrane proteins are participating.

Introduction

Scanning calorimetry provides a means for examining individual 'domains' within a complicated membrane structure, by focusing attention on specific

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structural transitions. Each of these transitions will be associated with a discrete set of components and/or a particular region of the total membrane, as was shown to be the case for the A transition [1]. Although the A transition is involved primarily with spectrin, an extrinsic membrane protein, certain other transitions could involve lipid domains or perhaps regions composed of interacting proteins and lipids. The study of these transitions would be of added importance if they occurred in regions of the membrane where critical functions are localized. Preliminary studies on erythrocyte membranes from seven other mammalian species show an unmistakable similarity of their transitions to those described here for human erythrocytes. This is at least a suggestion that they are associated with important structures whose integrity has been preserved by selective evolution. Further characterization of the transitions might then be useful in establishing important structural features and perhaps in elucidating structure-function relationships.

In the case of the erythrocyte membrane, which contains a cholesterol : phospholipid molar ratio of 0.9 [2], it is of particular interest to determine whether any of the phospholipids exist in a crystalline state in the native membrane. In the case of other membranes, it has frequently been suggested that local fluidity of a membrane might exert some control over function. Evidence is presented in this paper which is consistent with the existence of a small amount of highly-ordered lipid, even though the vast majority appears to be non-crystalline.

Experimental

Most of the materials and methods utilized in this work are identical to those described in other work [1,5,14].

In the preparation of papain-treated ghosts, the enzyme was incubated with Dodge ghosts for designated periods of time. The treated ghosts were then washed extensively to remove papain and any unbound fragments of membrane proteins.

Phospholipase A_2 (*Naja naja*) was purchased from Sigma Chemical Co. and used without further purification. Dodge ghosts were incubated with the enzyme in 0.1 M Tris buffer, pH 7.4, with 1 mM $CaCl_2$ for various times at room temperature with gentle stirring. 2 mM EDTA was then added to stop the reaction and the ghosts were washed three times with the above buffer. Finally, the ghosts were washed three additional times with 310 mosM phosphate buffer, pH 7.4.

Calorimetric studies were carried out in a differential scanning calorimeter described earlier [1,5], using matched 1.0 ml platinum cells. The scanning rate for most experiments was 40°/h and membrane concentrations were close to 1%.

Results

Effect of benzyl alcohol. Shown in curve 1 of Fig. 1 is the heat capacity profile for erythrocyte membranes suspended in 77 imosM phosphate buffer, pH 7.4, at a concentration of approx. 1% membrane. All of these transitions were

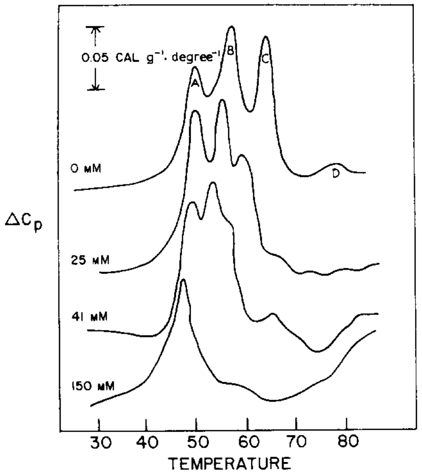


Fig. 1. The effect of benzyl alcohol on the calorimetric transitions of erythrocyte membrane. The buffer in each case was 77 imosM sodium phosphate, pH 7.4, and membrane concentrations were 0.6—1% by weight. The benzyl alcohol concentrations are shown and range from zero to 150 mM. Four transitions are seen, designated as the A (T_m = 50°C), B (T_m = 57°C), C (T_m = 63°C) and D (T_m = 75°C) transitions.

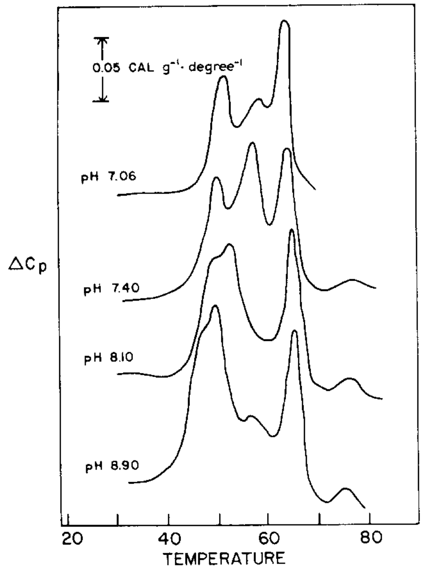
shown to be absent on the second heating, although some reversibility in the B transition could be achieved if the first heating was stopped at a temperature just above its midpoint [1]. Two of these transitions (B and C) were shown to be associated with very small CD changes [1] in the peptide-sensitive wavelength region and thereby could be due to processes other than protein unfolding. To explore the possibility of lipid involvement, we have examined the effect of a local anesthetic, benzyl alcohol, on the transitions of the erythrocyte membrane. Benzyl alcohol is known to cause a sharp depression in the temperature at which certain phospholipids undergo the gel-to-liquid-crystal transitions, apparently by binding to the head group region of the phospholipid in the melted state [3,4]. For example, the transition temperature of dipalmitoyl phosphatidylcholine is depressed by 5°C upon the addition of 40 mM benzyl alcohol. On the other hand, there is no evidence that benzyl alcohol, when present in concentrations below 50 mM, has a general effect in lowering the temperature at which protein denaturation might occur. It is reasonable then to expect that the addition of benzyl alcohol might provide some evidence as to which of these erythrocyte transitions involve significant phospholipid participation.

Erythrocyte ghosts in a number of solutions of different benzyl alcohol concentration (0, 20, 25, 41, 60, 100, 150, 200 mM), but otherwise identical conditions (77 imosM sodium phosphate, pH 7.4, approx. 1% membrane by weight), have been examined by heat capacity calorimetry. Examination of treated membranes by acrylamide gel electrophoresis showed no major changes in protein content caused by treatment with the alcohol. Some of the calorimetric results are shown in Fig. 1. Several trends are evident in these data. The progressive addition of benzyl alcohol acts to depress the transition temperature for both the B and C transition: the effect at low concentration being greater on C than on B. Concomitantly with the temperature shift, the transitions seem to broaden to some extent and to lose area. Because of this, it is not possible to see the B and C transition emerge on the low temperature side of A. The effect of benzyl alcohol on the midpoint of the A transition is small, as expected, since this transition is due to the thermal denaturation of spectrin and does not involve significant phospholipid participation under these conditions [1]. The fate of the D transition is uncertain. As benzyl alcohol is introduced into these suspensions, there is a significant increase in the thermal noise at high temperatures as well as rather erratic changes in the high temperature 'baseline', evident in the 41 mM and 150 mM samples in Fig. 1. Because of these complications and because of its small size, the D transition cannot be followed reliably.

More detailed studies (to be published) employing other amphipathic compounds give results similar to those employing benzyl alcohol. For example, various aliphatic alcohols are effective in shifting the transition temperatures of the B_2 and C transitions at low concentrations where they cause no measurable shift in the A transition temperature or the transition temperature for serum alumin denaturation. Certain drugs, such as chlorpromazine, show effects at much lower concentrations than seen for aliphatic and aromatic alcohols. At 40 μM, the C transition is shifted down by 4°C by chlorpromazine, with no measurable effect on the A, B_1, or B_2 transitions. This same concentration of chlorpromazine also has no measurable effect on the transition temperature for serum albumin denaturation. These studies with amphipathic additives show that the C transition exhibits a very high sensitivity which is not seen for either the denaturation of spectrin or for the denaturation of serum albumin. The B transitions exhibit high sensitivity in some cases, but the pattern of response is less predictable than for the C transition.

The pH dependence of the thermal transitions at moderate (77 imosM) salt. In this study, eleven samples at pH values ranging from 6.0 to 9.7 were examined in the calorimeter. In all cases, the supporting electrolyte was the same, i.e., 77 imosM sodium phosphate buffer. Although the buffering capacity was fairly weak over certain parts of this pH range, it was noted that the pH difference between native and irreversibly heated membrane suspensions was always small (approx. 0.1—0.2 pH units). A portion of the calorimetric data is shown in Fig. 2. The most striking trend is that the B transition shifts very strongly toward lower temperatures in the pH region 7.4—8.1. Close inspection also shows that the C transition undergoes a much smaller shift to higher temperature over the same pH region. Below pH 7.2, the B transition seems to shrink in size, as can be seen in the pH 7.06 sample. At pH 6.5 and 6.0, neither the B nor the C transition are well defined (not shown) since the calorimeter curve is broad and structureless in the 50—65°C region. Finally, at pH 8.9 (Fig. 2) and 9.7, there is some evidence of a new small transition at approx. 57°C.

All of the pH data are summarized in Fig. 3, where the thermal midpoints of the A, B, and C transitions are shown as a function of pH. It should be emphasized that at pH 8.5 and above, there are some uncertainties in the curves for the A and B transitions since the two transitions are overlapping and it is not possible to tell for certain whether the high or low temperature shoulder belongs to A. However, because the two transitions are only 1—2°C apart anyway, this uncertainty is not critical for the present analysis. These data show that the B transition shifts to lower temperature by at least 8—10°C, with an



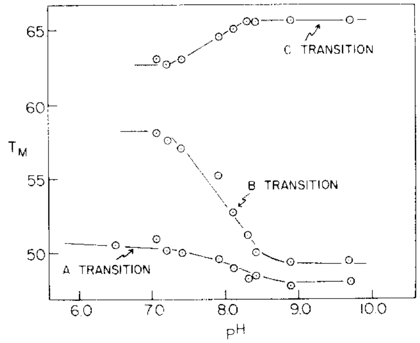


Fig. 2. The effect of pH on the calorimetric transitions at 77 imosM. The buffer and concentrations are the same as in Fig. 1. The pH was varied at constant osmolarity by mixing varying amounts of the mono- and di-basic forms prior to equilibration with the membranes.

Fig. 3. The variation of the transitions midpoints, T_m, with pH at 77 imosM.

apparent p*K* near 7.5—8.0. Because of the problems noted earlier in following the B and C transitions at pH values below 7, it is possible that we are not seeing the entire 'titration curve' here. Consequently, the exact p*K* for the effects seen and the total magnitude of the temperature change for titration are uncertain.

The change in the transition temperature for the A transition is very small over this pH range, as is that for the D transition (not shown). The effect of pH on the C transition (Fig. 3) is also much less pronounced than for B, but a definite increase in T_m occurs with an apparent p*K* identical to that of the B transition. The total change in the T_m amounts to only a couple degrees in this case.

Effect of buffer concentration and salt concentration. The results discussed up to this point have been for membranes in 77 imosM phosphate buffer. The calorimetric transitions change markedly as the concentration of buffer is increased. This was shown in an earlier paper [5], and has now been investigated in more detail. The scans in Fig. 4 show results obtained for samples at five different buffer concentrations ranging from 77 to 310 imosM and at a constant pH of 7.4. Several prominent changes can be seen to occur in going from solutions of low to high buffer concentrations. To begin with, both the A and C transitions become more prominent. This results from both a sharpening up of the transitions on the temperature axis along with some increase in area. This effect seems to occur mostly in the 77—155 imosM range and results in more than a doubling in the height of each heat capacity peak.

Of more interest is the process of alteration which is occurring in the B transition. As the buffer concentration is increased above 155 imosM, it can be

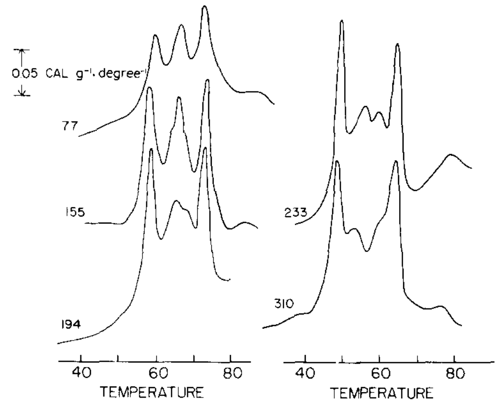


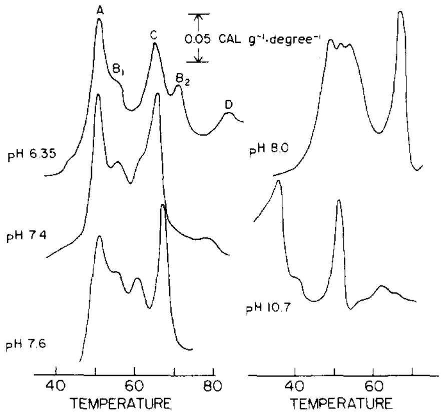
Fig. 4. The effect of buffer concentration on the calorimetric transitions. The pH in each case was 7.40 and the concentration of sodium phosphate buffer was varied from 77 to 310 imosM, as indicated.

seen that the B transition is splitting into two smaller transitions. One of these is ultimately localized (310 imosM) on the lower temperature side of the low-salt B transition and one on the high temperature side. We will refer to these two transitions as the B_1 transition and the B_2 transitions, respectively.

It is clear from these data that increased buffer concentration causes an 'uncoupling' of the B transition into at least two independent transitions. This seems not to be a specific effect of the particular buffer being used. The same pattern of changes occurs in sodium cacodylate as well, in going from 77 to 310 imosM at pH 7.4. It can also be brought about be adding either NaCl or KCl to a sodium phosphate buffer (77 imosM) in order to make a total ideal milliosmolarity of 310. Finally, divalent cations are considerably more effective in splitting the B transition than monovalent cations. In this regard, Ca^{2+} has been found in preliminary studies to be particularly effective even at concentrations of about 0.1 mM. In addition to splitting B into the B_1 and B_2 transitions, Ca^{2+} in higher concentrations has the effect of shifting the B_2 transition to higher temperatures and causing it to decrease in size (to be published). In contrast, the B_1 transition does not exhibit this sensitivity to low concentrations of Ca^{2+}.

The pH dependence of the thermal transitions at high (310 imosM) salt. It was shown earlier that the B transition exhibited an apparent p*K* near 7.5 under conditions of moderate ionic strength. Presumably this is due to the titration of a particular group or class of groups located in the cooperative structural domain which is involved in the B transition. At high ionic strength the B transition is split into two transitions. This then raises the question concerning the location of the group(s) of p*K* 7.5 under conditions where the B transition has split. Presumably, the titrating groups could be located entirely in either the B_1 or B_2 transition or they could be split between the two transitions.

In this study, thermal scans have been taken at 16 different pH values over the range 6—11 in 310 imosM sodium phosphate buffer. In addition, six pH values were examined over the range 9—11 at higher salt (1 M NaCl) in glycine/NaOH buffer. Some of the scans are shown in Fig. 5 while the midpoints for each transition, as they vary with pH, are shown in Fig. 6.



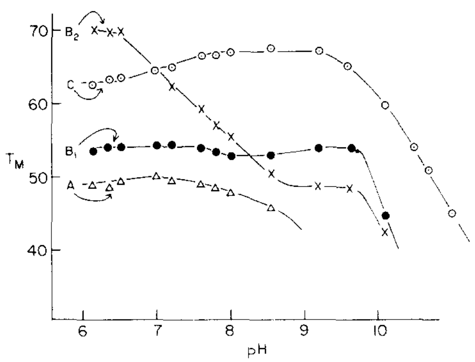


Fig. 5. The effect of pH on the calorimetric Transitions at 310 imosM. The sodium phosphate buffer was the same osmolarity in each case, while the pH was varied from 6.35 to 10.7 as indicated.

Fig. 6. The variation of the transition midpoints, T_m, with pH at 310 imosM.

Over the pH 6—9 range, the most obvious effect of pH is to shift the B_2 transition from about 70°C at pH 6 (where it is on the high temperature side of the C transition) to a value of about 50°C at pH 9. Thus, the B_2 transition shifts by about 20°C with an apparent pK near 7.5.

On the other hand, the B_1 transition shows essentially no change in thermal midpoint over the entire range from pH 6 to pH 9.5. The C transition shows a small increase in T_m over the same pH range where T_m for B_2 is decreasing, as it also did in the 77 imosM buffer (Fig. 3). The transition temperature for the A transition is nearly independent of pH until 8.5, where upon it begins to decrease with further increase in pH. However, the A transition cannot be studied reliably at pH values above about 9.5, since the area decreases as the pH becomes more alkaline. This might be due to the fact that spectrin is extracted from the membrane by exposure to alkaline pH [6]. Since our samples of ghosts were washed several times with the equilibrating buffer before scanning in the calorimeter, a substantial amount of the spectrin could be lost at the alkaline pH values.

Above pH 9.5, the C and B_1 transitions shift strongly to lower temperatures, as seen in Figs. 5 and 6. This happens in both the 310 imosM phosphate buffer and in the 1 M NaCl/glycine/NaOH buffer (not shown). It seems very likely that the B_2 transition also shifts down, but it is difficult to be positive on this point since the B_1 and B_2 transitions are broadening and losing area at these high pH values, as well as overlapping one another. The C transition, however, remains sharp and well defined even to pH 11, where its transition temperature is down to 45°C from a value of 67°C at pH 9.

From these results, it is clear that the group (or groups) which cause the B transition to shift with an apparent pK of approx. 7.5 at moderate ionic strength (77 imosM) becomes preferentially located in the B_2 transition at high ionic strength where the B transition is split. The T_m for the B_2 transition shifts by 20°C with an apparent pK of 7.5 under these conditions of high salt, while T_m for the B_1 transition is pH independent over the same pH range. It thus appears that the preferential segregation of the titrating groups into the B_2 transition is virtually 100% complete.

Reversibility studies of splitting of the B transition. The change in the thermal scan leading to a splitting of the B transition by electrolytes is probably related to changes in membrane structure which occur at low temperature as these electrolytes are added. The question might be asked as to whether or not these low-temperature structural changes are reversible provided that the membranes have not first been exposed to high temperature. In order to answer this question, we have prepared membranes under high salt conditions (310 imosM sodium phosphate buffer, pH 7.4; or with small amounts of Ca^{2+} in the suspensions) where the B transition is known to be split into two transitions. The buffer was then changed to conditions known to favor the unsplit B transition (77 imosM sodium phosphate buffer, pH 7.4). The samples were then heated in the calorimeter. It was found in these experiments that the calorimetric pattern observed depended only on the final conditions. That is, samples which had previously been exposed to high salt conditions and then taken to low salt conditions showed calorimetric scans which did not differ from the scans of membranes taken directly to low salt. It can be concluded from this that the low-temperature structural changes giving rise to the splitting of the B transition are reversible so long as there has been no exposure to high temperature.

Effects of proteolysis. Further information on the involvement of membrane proteins in the thermal transitions can be obtained by examining proteolized membranes in the calorimeter. External proteolysis of whole cells by trypsin is known to cleave only the glycoproteins [6] while a similar treatment by cymotrypsin results in the additional cleavage of band 3 to a 60 000 molecular weight fragment. Ghosts prepared from cells subjected to either trypsin cleavage or chymotrypsin cleavage were examined. Gel patterns were very similar to those reported by others [6]. The calorimetric scans of such proteolyzed membranes were found to be virtually identical to unproteolyzed membranes under the same conditions (Fig. 1, top scan) as long as no hemolysis had occurred. Thus, none of the transitions are sensitive to the cleavage of those portions of membrane proteins which are susceptible to external proteolysis.

The situation changes markedly if the proteolytic enzymes are allowed access to proteins exposed on the cytoplasm side of the membrane. Mild proteolysis of leaky ghosts with either trypsin or chymotrypsin first causes the disappearance of the B transition while more extensive treatment results in the additional loss of the A transition.

Shown in Fig. 7 is the gel scan of a membrane which has been subjected to the action of a relatively non-specific protease, papain. This particular sample was proteolyzed (leaky ghosts) for 2 h at a high papain concentration (0.10 mg/ml), followed by washings. By comparison with the unproteolyzed control, it can be seen that every major protein band has been cleaved by this treatment. Although some high molecular weight fragments can be seen in the gel scan, none of the original bands seem to be present to any great extent.

The papain-treated membrane was examined in the calorimeter, and the results are shown in Fig. 8. It can be seen from this scan that the A, B_1, B_2 and D transitions are greatly altered in this proteolyzed sample. There is a broad

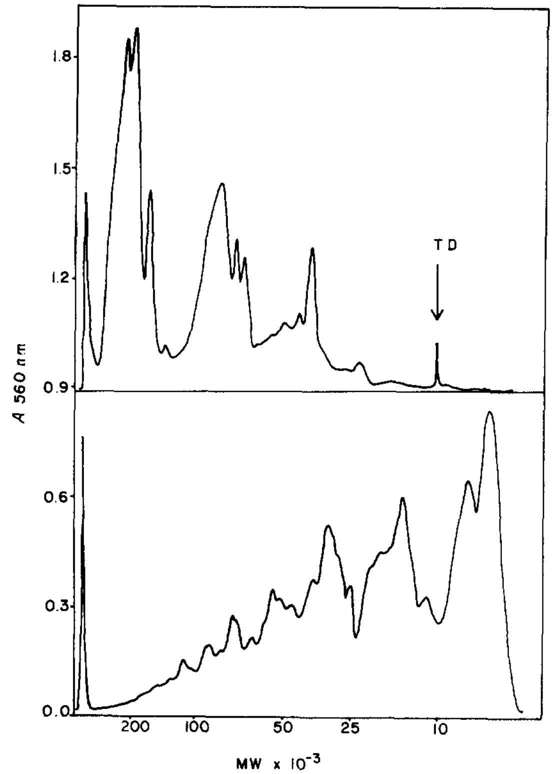


Fig. 7. Absorbance scans for SDS acrylamide gels stained with Coomassie Blue. (A). Untreated control sample. (B). Ghosts previously digested with papain (0.25 mg/ml) for 2 h, followed by extensive washing.

transition centered near 45°C, which is probably due largely to the unfolding of partially cleaved spectrin which remains attached to the membrane, i.e., the A transition. A poorly defined shoulder near 60°C might be the vestige of the B_2 transition. There is no indication of a B_1 transition remaining and the D

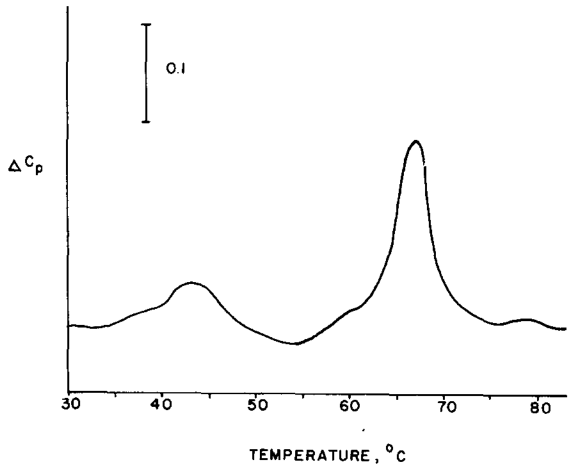


Fig. 8. Calorimetric scan for papain-treated ghosts. The sample was an aliquot of that shown in Fig. 7 (B). The suspending buffer was 310 imosM sodium phosphate, pH 7.4.

transition is essentially gone. In sharp contrast, the C transition is unaffected by this treatment both in terms of its size and its midpoint, which is at 66.5°C as in untreated ghosts. That this is truly the C transition is apparent from examining membranes exposed to shorter exposure times, since the 66°C transitions persists unaltered through all stages of proteolysis.

Effects of phospholipase A_2. Those transitions of the membrane which involve structural changes in lipid regions would be expected to be perturbed by extensive cleavage of phospholipids. In this study, leaky ghosts have been subjected to the action of phospholipase A_2 from *Naja naja.* This enzyme is known to cleave all of the glycerophospholipids in the ghost membrane [12], but does not cleave the sphingomyelin. Shown in Fig. 9 is the calorimetric scan for ghosts which had been previously treated for 120 min with a moderately high concentration (0.4 units/ml) of the enzyme at room temperature. Analysis of the phospholipids by thin layer chromatography showed no detectable amounts of phosphatidylserine, phosphatidylethanolamine or phosphatidylcholine and a normal amount of sphingomyelin. Examination of the treated ghosts by CD showed only very small differences from untreated ghosts after the spectra had been normalized to mean residue ellipticity. As can be seen, however, the calorimetric changes are very striking. Only the A transition is relatively unaffected, being of normal size and shifted lower in temperature by only 1°C. This might have been expected, since this transition involves the unfolding of an extrinsic membrane protein, spectrin. The B_1 transition is apparently absent in the treated membrane. The most striking alteration is the disappearance of the C transition. Studies involving shorter exposure times to phospholipase A_2 show that the C transition is the first to be modified, and begins to shift lower in temperature and decrease in size after only 1 min of incubation. Because the downward shift of C causes overlap with B_2, it is not possible at the present time to be certain of the fate of the B_2 transition. As seen in Fig. 9, a small transition remains at approx. 59°C after long incubation times. Although this temperature is nearly the same as that for the B_2 transition on native ghosts, there is no assurance that it is the B_2 transition. A conclusion on this point must await further studies. However, it is clear from these results

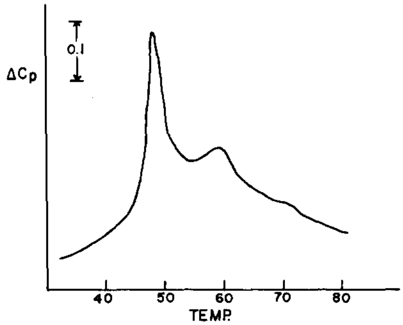


Fig. 9. Calorimetric scan for phospholipase-treated ghosts. Leaky ghosts were reacted with phospholipase A_2 (*Naja naja*) for 2 h, washed extensively, and resuspended in 310 mosM phosphate buffer, pH 7.4, for calorimetric analysis.

that the C transition and the B_1 transition are particularly susceptible to modification by phospholipase A_2.

The modification of erythrocyte transitions by phospholipase seems not to depend on the final cleavage products. Phospholipase C (*Bacillus cereus*) yields no lysophospholipids, but its action on ghosts produces essentially the same calorimetric pattern shown in Fig. 9 for the A_2 enzyme. Also, in some experiments the phospholipase A_2-treated membrane was extensively washed with buffer containing bovine serum albumin before calorimetric examination. This treatment removes all fatty acids and lysophospholipids from the membrane [11], but causes no further changes in the calorimetry.

Discussion

Presumably, each of the calorimetric transitions of the erythrocyte membrane must be associated with changes in structure of either proteins or phospholipids, or both. On the basis of the data now available, there is no evidence that extensive protein unfolding occurs in the B and C transitions, as it does in A and D. It was shown earlier [1] that very little change in CD could be observed for these transitions at 223 nM. Although it might be argued that protein denaturation could occur without significant change in the sensitive $n \rightarrow \pi^*$ peptide transition, this has never previously been reported to happen for any soluble protein and it would not a prior be expected for erythrocyte proteins where the average helical content is high (approx. 50%).

Also, these two transitions, particularly C, exhibit a high sensitivity to local anesthetics, as seen from the addition of benzyl alcohol and other compounds. These additives are known to be effective at low concentrations in depressing the melting temperature for lipid transitions. In fact, the temperature shift found [3] for vesicles of dipalmitoyl phosphatidylcholine is nearly the same as we have found for the C transition at comparable concentrations. There have been no detailed reports showing that benzyl alcohol, at low concentrations, will shift the transition temperature for protein denaturation by a comparable amount. In fact, we have shown that this additive has no significant effect on the denaturation of spectrin or serum albumin at concentrations which cause a shift of 8—10°C in the C transition. Hubbel et al. [7] did observe some changes in the spectra of attached spin labels as benzyl alcohol was added to lipid-free erythrocyte proteins at room temperature. However, the effects were seen only at alcohol concentrations of 50 mM and above and could have resulted from changes in protein aggregation rather than from protein denaturation.

Even though there is no evidence for the occurrence of protein unfolding, the proteolysis studies do suggest some type of protein involvement in or control over the B_1 and B_2 transitions. Although external proteolysis has no large effect on them, bilateral proteolysis causes their disappearance. This is an indication that the membrane regions involved in these transitions may be partially or completely localized on the cytoplasm side of the membrane. Fairly direct evidence obtained by a new method called thermal gel analysis (Brandts, J.F., Carlson, R., Taverna, R., Snow, J. and Lysko, K., unpublished, and ref. 13) shows that bands IV.1 and IV.2 are involved in the B_1 transition, band III involved in the B_2 transition and that all three of these integral proteins are involved in the coupled B transition at low salt. The evidence involves large changes in the gel migration of these specific proteins which occur sharply over the temperature range of these transitions, showing that changes occur in their state of aggregation. It, therefore, seems certain that protein molecules are somehow involved in those regions of the membrane which participate in the B transitions, even though there is as yet no evidence that they become extensively unfolded.

It could also be argued that the B transitions might involve phospholipid participation as well. This was suggested by their sensitivity to certain amphipathic additives. Also, cleavage with phospholipase A_2 resulted in the elimination of the B_1 transition, although the effect on B_2 is still unresolved. Thus, the B transition may be associated with fairly complicated regions of the membrane which would involve protein-protein, protein-lipid and lipid-lipid interactions.

One of the more interesting observations in this study has to do with the ability of the B transition to split into two smaller transitions. This occurs as the concentration of sodium phosphate buffer is increased above 77 imosM, or as monovalent salts such as sodium or potassium chloride are added. More strikingly, the splitting begins to occur at very low concentrations of Ca^{2+} (approx. 10^{-4} M Ca^{2+} in 77 imosM phosphate, pH 7.4). This behavior could be related to a similar observation by Jacobson and Papahadjopoulos [8], where splitting of a transition for mixed phosphatidylserine-phosphatidylcholine vesicles was observed as Ca^{2+} was added. In the latter case, the splitting was apparently brought about by lateral phase separation induced by the electrolyte.

Our studies give no evidence of protein involvement in the C transition. The lack of CD change and the complete resistance of the transition to extensive bilateral proteolysis support this idea. Its high sensitivity to amphipathic additives suggests the possibility that most of the enthalpy change in this transition could result from phospholipid melting. Evidence of a more direct nature comes from the phospholipase experiments, where the C transition was found to be most susceptible to the action of phospholipase A_2. Although more information is necessary, it is interesting to proceed a little further with the speculation. The enthalpy change, ΔH, for the C transition is in the range 0.5—1.0 kcal per mol phospholipid on the membrane. The uncertainty arises because of the difficulty in estimating areas for overlapping transitions. For comparison, gel-to-liquid-crystal transitions of model phospholipid vesicles exhibit ΔH values of 10—15 kcal/mol for those lipids with melting temperatures above 50°C [9]. The ratio of ΔH for the C transition to ΔH for phospholipid vesicles would suggest that only a small fraction of the total lipids in the membrane are participating in the C transition, i.e., something on the order 5%.

In summary, these studies are consistent with the idea that most of the phospholipids in the erythrocyte membrane exist in a non-ordered state, and thereby display no melting transition over the temperature range from 0° to 90°C. This is not unexpected in view of the high cholesterol content. However, the possibility cannot be ruled out that the C transition is due to the melting of a small fraction of the total phospholipid complement which exists in a highly-ordered state on the native membrane. The presence of even a small amount of lipid in crystalline patches on the membrane could be an important aspect of the overall structure, particularly if the organized lipids were close to or a part of functional sites. In this regard, it has recently been shown [10,14] that the C transition interacts very strongly with several highly specific inhibitors of anion transport.

The C transition is known to be nonreversible upon cooling and reheating [1]. This would be unusual for a lipid melting transition. However, it is conceivable that the reassembly of the crystalline region would be prevented because of the heat denaturation of certain proteins located close by in the native structure. It is also possible that lipid melting in general might be more difficult to reverse in a high-cholesterol membrane if the cholesterol partitions into the region subsequent to melting.

Our results are also compatible with other explanations for the C transition which do not involve lipid melting. For example, one might suppose the C transition to involve changes in interaction of those portions of integral membrane proteins not susceptible to proteolysis. These protein segments would be expected to be 'solvated' by lipids in the membrane and, hence, changes in protein-protein interactions might critically depend on protein-lipid interactions. The high sensitivity to phospholipases and to amphipathic additives might then be understandable in terms of lipid perturbations which in turn affect protein-lipid and protein-protein interactions.

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References

1 Brandts, J.F., Erickson, L., Lysko, K., Schwartz, A.T. and Taverna, R.D. (1977) Biochemistry 15, 3450

2 Van Deenen, L.L.M. and de Gier, J. (1975) in The Red Blood Cell (Surgenor, D., ed.), 2nd edn., Vol. 1, chapt. 2, Academic Press, New York

3 Colley, C.M. and Metcalfe, J.C. (1972) FEBS Lett. 24, 241

4 Metcalfe, J.C. (1975) in Functional Linkage in Biochemical Systems (Schmidt, F.O., Schneider, D.M. and Crothers, D.M., eds), p. 90, Raven Press, New York

5 Jackson, W.M., Kostyla, J., Nordin, J. and Brandts, J.F. (1973) Biochemistry 12, 3662

6 Steck, T. (1972) in Membrane Research (Fox, F., ed.), p. 71, Academic Press, New York

7 Hubbell, W.L., Metcalfe, J.C., Metcalfe, S.M. and McConnell, H.M. (1970) Biochim. Biophys. Acta 219, 415—127

8 Jacobson, K. and Papahadjopoulos, D. (1975) Biochemistry 14, 152

9 Ladbrooke, B. and Chapman, D. (1969) Chem. Phys. Lipids 3, 304

10 Snow, J.W., Brandts, J.F. and Low, P.S. (1977) Fed. Proc. Abstr. 1977 FASEB Meet., in the press

11 Roelofsen, B. and van Deenen, L.L.M. (1973) Eur. J. Biochem. 40, 245

12 Zwaal, R. and Roelofsen, B. (1976) in Biochemical Methods of Analysis (Maddy, A.H., ed.), p. 352, Chapman and Hall, London

13 Brandts, J.F., Lysko, K., Schwartz, A.T., Erickson, L., Carlson, R., Vincentelli, J. and Taverna, R.D. (1970) Colloques Internationaux du C.N.R.S., No. 246, L'eau et los Systems Biologiques, 169—175

14 Snow, J., Brandts, J.F. and Low, P. (1978) Biochim. Biophys. Acta 512, 579—591