Calorimetric Studies of the Structural Transitions of the Human Erythrocyte Membrane. The Involvement of Spectrin in the A Transition†

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ABSTRACT: Using scanning calorimetry and circular dichroism (CD), the four structural transitions of erythrocyte ghosts have been examined in more detail. It is shown that only one of these, the B transition, exhibits any degree of reversibility and then only if exposed to fairly low temperature for short times. The A and D transitions are accompanied by large changes in CD in the 220-nm region while the B and C transitions are not, an observation consistent with other evidence suggesting that the B and C transitions may not involve extensive protein unfolding. It is also shown that the A transition is readily removed from the membrane by the extraction of spectrin, while the other three transitions are left on the membrane nearly unchanged. The A transition is intact in the supernatant containing soluble spectrin, and the transition temperature is unchanged from that exhibited by the native membrane. Soluble spectrin was found to have a CD spectrum which is extremely similar to that of muscle myosin. This tends to support previous suggestions that the two proteins might be structurally related. The results of this study are consistent with the idea that the spectrin molecule contains two or more structurally independent regions and that only one of these regions "melts out" in the A transition.

In a previous report from this laboratory, calorimetric experiments on human erythrocyte membranes were described (Jackson et al., 1973). It was shown that these membranes display four well-defined structural transitions over the temperature range 45 to 80 °C. The results described in the present paper provide a more detailed experimental description of these interesting transitions. It is shown that perhaps only two of the four transitions are due to simple protein unfolding reactions. One of these denaturation transitions can be removed from the membrane by exposure to low salt, so it seems certain that it is due to the partial unfolding of the spectrin complex. Studies carried out on the extracted protein tend to confirm this idea, and further suggest that the spectrin complex is a highly helical protein which consists of two or more independently folded regions.

Experimental Section

Membranes were prepared from freshly drawn human blood by the method of Dodge et al. (1963). The final composition of the suspending buffer was attained by washing packed cells several times with a large excess volume. Membrane concentrations were determined by drying to constant weight at 105 °C. Protein concentrations were routinely determined on both membrane samples and solubilized spectrin by the method of Lowry et al. (1951).

Molecular weight profiles of membrane proteins were obtained on acrylamide gels, using the method described by Fairbanks et al. (1971). Samples were electrophoresed on 5.6% gels in a buffer containing 0.2% sodium dodecyl sulfate.

The spectrin extract was prepared by mixing equal volumes of packed ghosts in 20 mosm sodium phosphate buffer, pH 7.4, with pH 9.0 solution containing 1 mM EDTA[1] (Hoogevan et al., 1970). This mixture was then placed in a dialysis bag and incubated with a large excess of the EDTA extracting solution for 48 h at 5 °C. The extracted membrane was separated from the supernatant by centrifuging at 10 000*g* for 40 min. The supernatant was then centrifuged at 90 000*g* for 1 h to separate the soluble spectrin from small vesicles found to be present.

Heat capacity measurements were made in a specially constructed differential calorimeter (Jackson and Brandts, 1970; Jackson, 1970) using methods described earlier (Jackson et al., 1973). The concentration of membranes was normally about 1% by weight, while the concentration of spectrin was ca. 0.2% for samples examined in the calorimeter.

Circular dichroism measurements were made on a Cary 61 spectropolarimeter, using a thermostated cell of either 0.1 or 1.0 mm. For the experiments where temperature was used as a variable, the heating rate was very close to 20 °C/h. This was attained by using a Neslab temperature programmer connected to a conventional bath with a mercury-contact regulator. The cell temperature was determined in a calibration run, where a thermocouple was immersed directly in the light path. The cell temperature was measured directly as a function of bath temperature at a heating rate of 20 °C/h.

Results

Detailed Studies of Reversibility. Using differential heat capacity calorimetry, four thermal transitions can be readily seen in dilute (ca. 0.5% protein) suspensions of ghosts. When the scanning rate of the calorimeter is ca. 20 °C/h, the transitions are well separated on the temperature axis and centered at 49.5, 57, 63.5, and 75 °C at pH 7.4 and 77 mosm sodium phosphate buffer. This is shown in Figure 1, curve 1. These transitions will be referred to as the A, B, C, and D transitions, in order of increasing transition temperature. The thermal midpoints of the three large transitions are reproducible within ca. 0.5 °C from sample to sample. The magnitudes (i.e., areas)

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; mosm, milliosmolar.

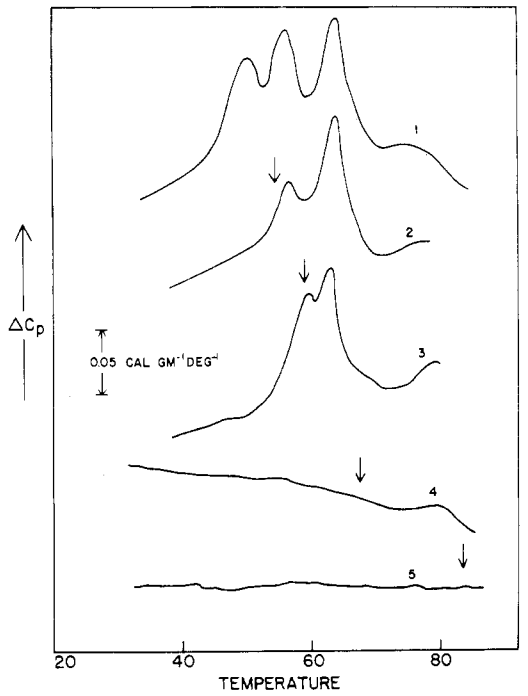

FIGURE 1: Reversibility studies of the calorimetric transitions. The buffer used for each of the five samples was the same (77 mosm sodium phosphate, pH 7.40). The membrane concentrations were 0.6–1.0% by weight. Curve 1 shows the heat capacity vs. temperature for the entire first heating under these conditions. Curves 2–5 show second heatings in the calorimeter, for samples which had been heated to different temperatures during the first heating. In each case, the arrow designates the temperature at which the first heating was stopped prior to cooling and reheating. The scanning rate was ca. 20 °C/h in all cases.

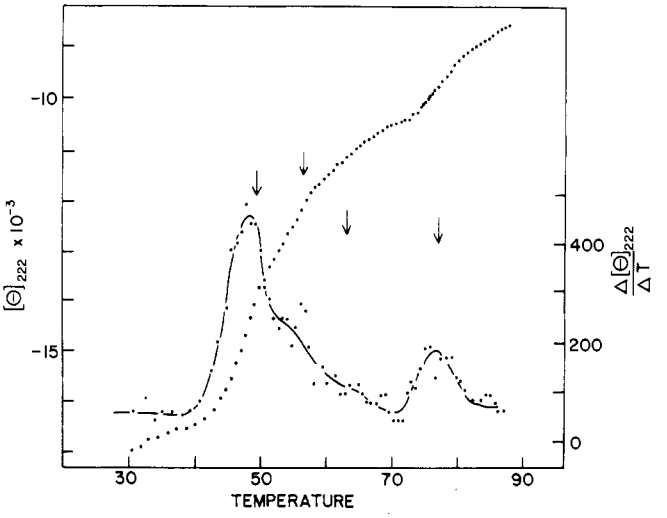

FIGURE 2: The temperature dependence of circular dichroism for erythrocyte ghosts. The conditions were identical with those of Figure 1 except the membrane concentration was 0.04% for this experiment, which utilized a 1.0-mm jacketed cell. The scanning rate was ca. 20 °C/h. The mean residue ellipticity was calculated using an average residue weight of 114, and is shown on the left ordinate. The thermal variation parameter on the right ordinate was calculated from differences in the mean residue ellipticity over temperature intervals of 1.25 °C. The arrows designate the midpoints of the A, B, C, and D transitions as seen in the scanning calorimeter under identical conditions and with the same scanning rate as employed in the CD studies.

of the transitions are not so reproducible since 10–15% variations have been noted. These depend not so much on the donor, but more on small differences in other variables which have not been well characterized in this study (i.e., age of cells, age of ghosts, number of washes, etc.).

The reversibility of these transitions has been examined in four separate experiments illustrated in Figure 1, curves 2 through 5. In each of these experiments a fresh sample of ghosts was initially heated in the calorimeter to the temperature indicated by the arrow in each of the curves. The calorimeter was then cooled quickly back to low temperature and the sample heated a second time. The traces shown in curves 2–5 are the heat capacities measured during the second heating. The heat capacity changes for the first heating are not shown but were generally in good agreement with curve 1 up until the temperature where the first heating was stopped.

It is seen that if a sample is heated through the A transition on the first heating (curve 2), then the second heating shows nearly no A transition, although the B, C, and D transitions are nearly normal. If the first heating is taken through both the A and B transitions (curve 3), then the second heating lacks the A transition but shows normal C and D transitions, as well as giving some indication of partial reversibility of the B transition. However, the B peak seen on the second heating is about 2.5 °C higher than expected for a completely reversible B transition (cf. curve 1), even though the area associated with this modified B transition is fairly large. If, on the other hand, the first heating passes through the A, B, and C transitions (curve 4), then none of the three large transitions are seen on the second heating, i.e., there is virtually total irreversibility in this case. As might be expected, the same is true if the first heating passes through the D transition (curve 5).

We conclude from this that these transitions all show a tendency to be irreversible under the conditions which were used in these experiments. In the case of the A, C, and D transitions, the irreversibility is virtually complete. However, the B transition shows some tendency for reversibility providing the first heating passes only through the B transition and not through the C transition. This conditional reversibility pattern suggests a possible interaction between the structural regions involved in the B and C transitions.

Comparison of Calorimetric and Circular Dichroism Changes. Because of their irreversibility, the temperature at which the transitions occur will depend on the heating rate. In order to precisely compare calorimetric data with data obtained by other techniques, the same heating schedule must be used in all cases. Consequently, the circular dichroism (CD) data reported here were obtained on samples which were being heated at a rate nearly identical with the heating rate for the scanning calorimeter, i.e. 20 °C/h.

The circular dichroism spectrum of native erythrocyte ghosts (Lenard and Singer, 1966; Gordon and Holzworth, 1971) contains minima at 222 and 207 nm and a maximum at ca. 190 nm, a pattern which presumably reflects the high content of α helix in the membrane proteins. One point of interest with regard to the four membrane transitions is whether any or all of them are due to the unfolding of membrane-bound protein. If this is the case then large cooperative changes in the CD signal should be seen. If, on the other hand, all or certain of the four transitions are not predominantly reflecting protein unfolding, then less change in CD would be expected over the temperature range of these transitions.

The results of the CD study are shown in Figure 2, where the magnitude of the ellipicity at 222 nm is plotted (dotted curve) as a function of temperature for a suspension of ghosts in 77 mosm sodium phosphate buffer (pH 7.4). In order to permit a better comparison with the heat capacity data, the variation parameter $\Delta\theta/\Delta T$ is also plotted as the solid curve.

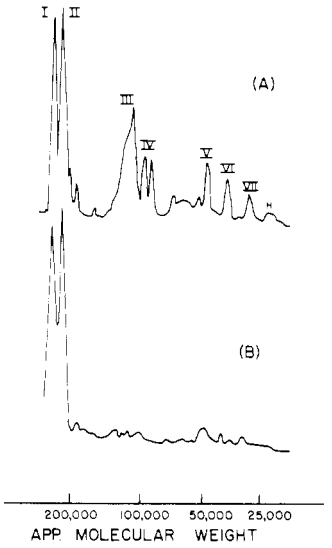

FIGURE 3: Sodium dodecyl sulfate–acrylamide gel patterns for erythrocyte membrane proteins. The gels were from 5% acrylamide and were run in the presence of 0.2% sodium dodecyl sulfate and 0.040 M dithiothreitol: (A) unmodified erythrocyte ghosts; (B) spectrin which has been extracted by exposure of ghosts to desalted solvent, pH 9, 0.001 M EDTA for 2 days at 4 °C.

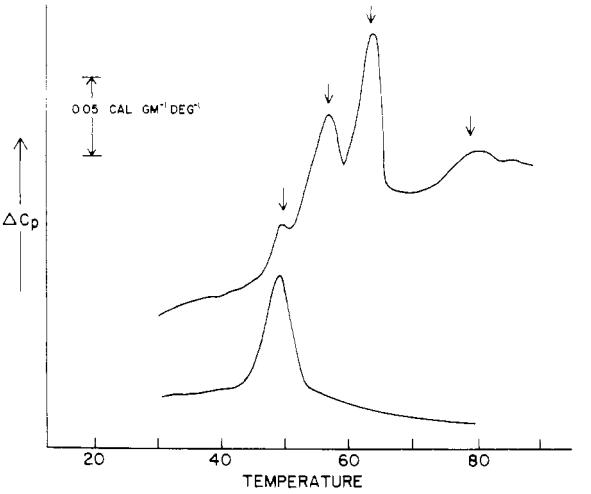

FIGURE 4: Calorimetric scans for spectrin and for spectrin-depleted membranes. All conditions are identical with those of Figure 1. The upper curve shows the calorimetric scan for spectrin-depleted membranes while the lower curve is the scan for the extracted spectrin. The arrows designate the midpoints of the A, B, C, and D transitions for unmodified membranes under identical conditions.

The midpoints of the A, B, C, and D transitions in the calorimeter (data of Figure 1, curve 1) under these same conditions are indicated with arrows. It is seen from this comparison that the A transition leads to rather substantial changes in CD and that these occur in a highly cooperative manner. There is only the suggestion of a small shoulder near 57 °C where the B transition occurs in the calorimeter while no well-defined CD transition is apparent over the temperature interval associated with the C transition. In view of its small thermodynamic size (cf. Figure 1), the D transition leads to a very large and cooperative loss in the apparent helical content.

These data argue against the idea that each of these four transitions is a manifestation of essentially similar structural processes. In view of the CD results, it is likely that only transitions A and D are associated with large changes in protein helix. The enthalpy change in the B and C transitions would appear then not to result primarily from the unfolding of membrane-bound proteins, although we can certainly not rule out the possibility that some unfolding does occur in these latter transitions. Even so, the very small ratio of CD change/enthalpy change is most probably an indication that structural modifications other than protein unfolding are involved in the B and C transitions.

The results in Figure 2 also show that the membrane contains at least some proteins which possess very stable helices. Even at a temperature of 90 °C, the value of $(\theta)_{222}$ is still about 50% of its value at 30 °C. It will be seen later that the spectrin molecule is probably one of the membrane proteins which has some unusually stable helical structure that persists even at very high temperatures.

Extraction of the A Transition from the Membrane. When ghosts are suspended in deionized water with 0.001 M EDTA (pH 9) a portion of the membrane protein is solubilized (Hoogevan et al., 1970; Marchesi et al., 1969; Clarke, 1971). This protein extract is called spectrin and it can be separated from the residual spectrin-poor membrane by centrifugation. Inspection of sodium dodecyl sulfate–polyacrylamide gels confirms that the above procedure results in the solubilization of protein bands I (mol wt 240 000) and II (mol wt 200 000), using the terminology of Steck (1972). This is shown in the gel scan of Figure 3. Invariably, some band V (mol wt 40 000) is also extracted along with smaller amounts of other proteins. The extract is usually pink in color and contains some hemoglobin, although gel scans show this to be a relatively minor contaminant of ca. 5% or less, by weight.

Following extraction, the solubilized spectrin was separated from the residual, spectrin-poor membrane by centrifugation. Both of these samples were then dialyzed to pH 7.4, 77 mosm phosphate buffer (i.e., the conditions of Figure 1) and examined individually in the calorimeter. Shown in the top curve of Figure 4 is the thermal scan of residual membranes from which spectrin has been extracted. The arrows indicate the transition temperatures expected for an unmodified membrane under these same conditions (curve 1, Figure 1). It is clear that the major change which results from spectrin extraction is the nearly complete loss of the A transition. The B, C, and D transitions are all of about normal size and occur at the same temperatures as in the native membrane. The small amount of the A transition remaining on the membrane is probably caused by the inability to extract more than about 80% of the spectrin with the mild extraction conditions used here.

Since the A transition is missing from the extracted membrane, we might expect to find it in the solubilized spectrin if that protein is still in its native structure. This turns out to be the case. The lower curve of Figure 4 shows the calorimetric scan on solubilized spectrin. This protein exhibits a single well-defined transition with a midpoint at 49 °C. This is, within error, the same temperature where the A transition is observed for unmodified ghosts under the same solution conditions. Furthermore, the amplitude of the exothermic peak (from low-temperature baseline to peak maximum) is ca. 0.45 cal/deg per g of spectrin. If one assumes that it is also components I and II in the native ghost which give rise to the A transition and if one further assumes that spectrin comprises 30% of the total ghost protein (ca. 15% of the total ghost weight), then the amplitude of the A transition on the native membrane (Figure 1, curve 1) is 0.50 cal/deg *per g of spectrin* on the native membrane. The agreement between these two amplitudes provides further support for the idea that the A transition in

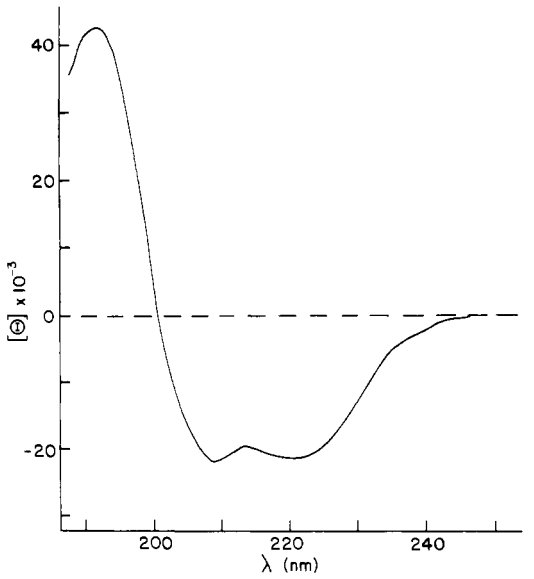

FIGURE 5: The circular dichroism spectrum for extracted spectrin. The buffer conditions are identical with those of Figure 1. The mean residue rotation was calculated using an average residue weight of 114. Data were obtained at 25 °C in a 0.1-mm cell.

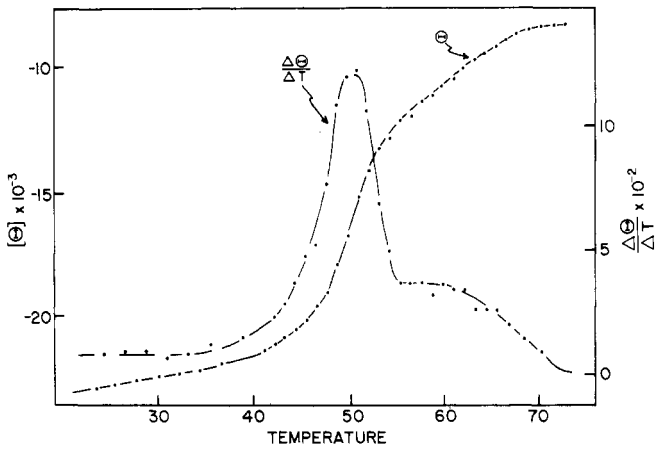

FIGURE 6: The temperature dependence of circular dichroism for extracted spectrin. The buffer is the same as in Figure 1. Data were obtained in a 1.0-mm jacketed cell at a scanning rate of 20 °C/h. The mean residue rotation (left ordinate) and the variation parameter (right ordinate) were obtained as in Figure 2.

native ghosts and the transition observed for soluble spectrin are, in fact, the same process.

It is difficult to estimate ΔH (i.e., the integrated peak area in Figure 1) involved in the A transition on the membrane due to overlap with B. It does appear to be approximately the same as for the solubilized spectrin on a per gram basis. The ΔH for the transition of solubilized spectrin can be estimated from Figure 4 using the method of Jackson and Brandts (1970) and is found to be close to 2.0 cal/g. Although this estimate contains moderately large uncertainties, it still seems likely that ΔH for spectrin denaturation is much smaller than commonly observed for small globular proteins at the same temperature (ca. 5 cal/g for chymotrypsinogen (Jackson and Brandts, 1970) and 7.5 cal/g for ribonuclease (Jackson, 1970)). This suggests the possibility that the "unfolding" process for this high molecular weight erythrocyte protein may not be as complete as is frequently observed for the reversible denaturation of low molecular weight globular proteins. Further evidence to support this contention comes from CD experiments described below.

Circular Dichroism Studies on Solubilized Spectrin. The application of the circular dichroism technique to membrane suspensions has been of only limited value, due in part to artifacts introduced by light scattering and absorption flattening (Urry, 1972; Wallach and Winzler, 1974). These artifacts will not be present for solubilized membrane proteins and this has prompted us to carefully examine the optical activity of extracted spectrin. The circular dichroism spectrum of native spectrin (25 °C) is shown in Figure 5. The characteristic helical pattern, with minima at 222 and 209 nm and with a large maximum at 190 nm, is clearly evident. The value of $(\theta)_{222}$ of −21 000 indicates a substantial helical content since the completely helical forms of (glutamic acid) or polylysine have corresponding values of $(\theta)_{222}$ of only about −39 500. This is in keeping with numerous suggestions that the spectrin complex is a rodlike molecule with considerable helix (Marchesi et al., 1969; Clarke, 1971; Singer, 1974). It has been speculated, in fact, that spectrin may closely resemble muscle myosin (Singer, 1974). It is interesting then that the CD spectrum of spectrin shown in Figure 6 is extremely similar, over the entire wavelength range, to that of rabbit muscle myosin. That protein also has characteristic extrema at 222, 209, and 191 nm with ellipticities of −23 000, −22 000 and 48 000, respectively (Oikawa et al., 1968) relative to corresponding values of −21 000, −22 500, and 43 000 for spectrin.

When soluble spectrin is heated at 20 °C/h, a transition in the CD bands is seen at the same temperature as the calorimetric transition previously described (Figure 4, lower curve). This is shown in Figure 6, where both the ellipticity and the temperature derivative of ellipticity are plotted vs. the temperature. The transition in the CD is centered at 50 °C and it gives rise to substantial changes in ellipticity. The reversal point obtained after cooling to 25 °C shows that the transition is not reversible, as do reheating experiments. Over the temperature range from 40 to 55 °C where the transition takes place the value of $(\theta)_{224}$ decreases by less than 40%. Thus, the spectrin complex still has a substantial helical content even after the cooperative transition. This possibility was suggested earlier by the calorimetric behavior of spectrin, as reflected in the small magnitude of ΔH.

The size of the CD change for soluble spectrin is consistent with the idea that the structural changes occurring during the denaturation are nearly the same as those which occur during the A transition on the native membrane. Again assuming that ca. 30% of the membrane protein is spectrin, the data of Figure 6 would lead to the prediction that the change in $\Delta\theta/\Delta T$ would be about 350 from the low-temperature side of the peak to the maximum for the native membrane. The actual value measured for the A transition on the native membrane is about 425 (Figure 2).

More careful scrutiny of the data of Figure 6 indicates that the spectrin complex may actually be undergoing two transitions, with a small broad process centered near 60 °C and forming a shoulder on the major peak in the derivative plot. There is some indication that this shoulder might also be present on the high-temperature side of the A transition of the native membrane (cf. Figure 2), although it is more difficult to decide with certainty due to the proximity of the B transition. At any rate, the ellipticity for soluble spectrin becomes nearly independent of temperature above 70 °C. At these high temperatures, the ellipticity value of −8000 suggests the presence of some structure which is extremely resistant to thermal unfolding.

Discussion

In an earlier paper, it was suggested (Jackson et al., 1973) that all four of the erythrocyte transitions might involve extensive protein unfolding. The more detailed studies reported here show, however, that most of the cooperative CD change is localized in the A and the D transitions. Although not conclusive, this finding does suggest that the other two transitions may not involve large amounts of protein unfolding. Additional evidence will be presented in a later paper showing that the B and especially the C transitions, even though irreversible, display certain characteristics expected for order-disorder transitions of phospholipids while the A and the D transitions do not. Thus, our earlier suggestion that each of the transitions might be due *primarily* to protein unfolding now seems likely to be in error.

The localization of the major CD changes in the A and D transitions does support the idea that substantial protein unfolding occurs in at least two cases. Although phospholipids may display rather large CD bands near 190 nm (Chen and Kane, 1975) there are no reports of additional large bands in the 220–240-nm region where these studies were conducted. Thus, there is no reason to suppose that the CD changes in the A and D transitions are not caused by changes in protein conformation. The fact that the CD change for solubilized spectrin is nearly the same as for the A transition on the membrane also argues convincingly against the idea that phospholipids are major contributors to the change.

The particular proteins which might be involved in the denaturation associated with the D transition are not at all evident from these studies. Apparently, it is not hemoglobin since we have found that the D transition persists even in very white ghosts. This transition may involve the unfolding of a very helical structural region since the ratio of the CD change to the enthalpy change is very high (ca. 3–5 times higher than for the A transition).

The nature of the A transition has been more clearly elucidated in these studies. The extraction experiments provide strong evidence that the spectrin complex on the membrane is responsible for the A transition. The change in CD suggests a loss of secondary structure. It is a surprise that the A transition occurs at nearly the same temperature for solubilized spectrin as for spectrin on the native membrane. The most obvious interpretation of these findings is that the structural region of spectrin which unfolds in the A transition does not interact directly with the membrane. No shift in T_m would then be expected to occur when spectrin is dissociated. This idea is consistent with other observations which show that the A transition involves only a limited region of the total spectrin molecule, since the normalized enthalpy change for the A transition of soluble spectrin is rather small and since less than half of the apparent helical content of spectrin is lost in the A transition. Thus, it is possible that another structural region (or regions) is involved in anchoring the protein to the membrane and that this second structural region is not cooperatively modified in the A transition. If true, this suggests a "subunit" structure for spectrin. Other large protein complexes, such as myosin, are known to possess independent or semi-independent structural regions.

There is one piece of evidence which argues that there might be at least an indirect interaction between the A region of spectrin and other components of the membrane. Rakow and Hochmuth (1975a,b) have shown that there is a "transition" in the elastic properties of the erythrocyte membrane which is centered very close to 49 °C at pH 7.4, 260 mosm. This ultimately results in sphering, budding, and fragmentation of whole cells, according to them. We have observed independently that these same changes occur for ghosts being heated at 20 °C/h, and that the onset of these changes is very close to the center of the A transition. If there is a cause-and-effect relationship between the A transition and these changes in macroscopic membrane properties, as seems likely, then the A region of spectrin may indeed play an important role in maintaining membrane structure.

These results then suggest no direct interaction between the A region and the membrane but also show that the A region is somehow involved in maintaining the mechanical properties of the membrane. There is at least one simple way in which these two observations can be reconciled. One might consider the analogy of spectrin to a coiled spring, where the maintenance of mechanical properties requires tension in the spring. The function could then be impaired either because of disruption of the coil or because the hook attached to the load has broken. We would suggest that the A region of spectrin is analogous to the coil of the spring rather than the hook.

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