Journal of Cellular Biochemistry 30:361–370 (1986)

# Spectrin Involvement in a 40°C Structural Transition of the Red Blood Cell Membrane

**Maurizio Minetti, Marina Ceccarini, Anna Maria M. Di Stasi, Tamara C. Petrucci, and Vincent T. Marchesi**

*Laboratorio di Biologia Cellulare Istituto Superiore di Sanità, 00161 Rome, Italy (M.M., M.C., A.M.M.D.S., T.C.P.) and Department of Pathology, School of Medicine, Yale University, New Haven, Connecticut (V.T.M.)*

Proteins involved in a structural transition detected in red blood cell membranes at 40°C by spin labeling methods have been investigated. Antibodies specific for spectrin, band 3, and protein 4.1 have been used as specific probes to modify membrane thermotropic properties. Spectrin seems to be involved in a 40°C transition detected in ghosts by both a stearic acid spin label (16-doxyl stearic) and a sulfhydryl-specific maleimide analogue spin label. Circular dichroism and maleimide spin labeling studies of purified spectrin show a slow unfolding of the protein structure starting at 25–30°C and a massive transition with an onset temperature of 48 and 40°C, respectively. This thermotropic behavior of spectrin could be the process that modifies membrane physicochemical properties above 40°C that are detected by the stearic acid spin label. The transition detected by the stearic acid spin label was modified both by antispectrin antibodies and anti-4.1 protein antibodies, but not by antibodies specific for the cytoplasmic domain of band 3. These results suggest an involvement of protein 4.1 in regulating spectrin unfolding at the membrane level. A selective inhibition of the transition detected by the maleimide spin label has been obtained with a monoclonal anti-spectrin antibody at 1:1 molar ratio. The involvement in this transition of a localized spectrin domain(s) containing few exposed sulfhydryl groups is proposed.

**Key words: spin labeling, red blood cell structural transitions, spectrin, spectrin-membrane interaction, antibodies specific for erythrocyte proteins, cytoskeleton**

In the 0–50°C temperature range, red blood cell (RBC) membranes present discontinuous changes in some physical parameters that may be indicative of struc-

Abbreviations used: RBC, red blood cell; Abs, antibodies; 16 DSA, 2-(14-carboxy-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl; 4MSL, 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl; EPR, electron paramagnetic resonance; CD, circular dichroism; anti-80K, Abs specific for the 80K aminoterminal domain of $\alpha$-spectrin subunit; auto-I, monoclonal Abs obtained from a mouse with an autoimmune disease; anti-$\beta_I$, Abs specific for the $\beta_I$ domain of $\beta$-spectrin subunit; DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetracetic acid.

Received June 12, 1985; revised and accepted October 11, 1985.

tural transitions of membrane components. To study thermotropic properties of RBC membranes, spectroscopic techniques have been applied including those using labeled molecules as probes [1–3], and those considered to be unperturbing [4–6]. The rather good agreement between critical temperatures and the different membrane areas investigated by probes suggests that both proteins and lipids are involved in the observed thermotropic changes. In addition many recent studies implicate a convincing role for skeletal proteins in the topological organization of RBC membrane [7]. It is conceivable that such skeletal proteins modulate membrane properties through their interactions with the lipid bilayer. In this context it is interesting to note that the lateral mobility of band 3, the major intrinsic protein of RBC membrane, is slower than predicted by the viscosity of the local lipid environment [8] and is believed to be controlled by the cytoskeleton [9]. The rotational motion of spin-labeled sialic acids of glycophorin also seems to be affected by spectrin depletion from ghosts [10].

In a previous paper we presented evidence for a role of skeletal proteins in a transition detected at 40°C by a 16-doxyl stearic acid (16 DSA) spin label [11]. In the present work we investigated the effects of polyclonal and monoclonal antibodies (Abs) specific for some of the major RBC membrane proteins. Thermotropic properties of RBC membranes were monitored both with 16 DSA and with a maleimide spin label covalently bound to proteins. Our results confirm and clarify the role of skeletal proteins in this transition by showing a direct involvement of spectrin. A temperature-dependent unfolding of spectrin could be the process affecting membrane physicochemical properties above 40°C. Moreover, this approach has been useful to demonstrate a role of protein 4.1 in the 40° transition, thus suggesting a functional interaction with spectrin.

## MATERIALS AND METHODS

### Materials

2-(14-carboxy-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyl-oxyl (16 DSA) and 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (4MSL) were obtained from Syva (Palo Alto, CA). Diisopropylfluorophosphate (DFP) was obtained from Fluka (Buchs, Switzerland).

**Membrane Preparation and Reaction with Antibodies.** Unsealed ghosts were prepared from fresh human RBC with 5 mM phosphate buffer, pH8, at 0°C. To avoid proteolytic degradation 1 mM DFP and 1 mM EDTA were present during all the manipulations. The correct amount of Abs was calculated on the basis of data reported by Goodman and Shiffer [12]. Abs were dissolved at 0.5–1 mg/ml in 5 mM phosphate buffer, pH 8, and added to unsealed ghosts maintained at 0°C. After 10 min of incubation at the same temperature to allow Abs penetration inside the membranes, ghosts were resealed at 37°C with a buffer containing 150 mM KCl, 5 mM phosphate, 0.25 mM ATP, 0.25 mM $MgCl_2$, pH 7.2, and the above-mentioned protease inhibitors. Membranes were checked for permeability to macromolecules before and after the resealing by using fluorescent dextrans as previously described [3]. To minimize differences between RBC samples the comparison between control ghosts and Abs-treated ghosts was carried out with the same sample on the same day.

### Antibodies

Polyclonal Abs specific for band 3 and protein 4.1 were obtained and purified by using standard techniques and their specificity was demonstrated by immunoblot

analysis of both intact protein 4.1 and specific chemical domains prepared by controlled proteolytic digestion [13]. Monoclonal antibodies against the α-I domain of human erythrocyte spectrin were described previously [14]. Rabbit monospecific polyclonal antispectrin Abs were purified on a spectrin-Sepharose 4B column according to Nicholson and Painter [15].

**Spin labeling and EPR spectra analysis.** Spin label incorporation of 16 DSA into RBC membranes was performed by overnight incubation at 0°C. To 1.8 μg of dried probe an amount of pelleted membranes corresponding to 0.3 mg of lipids was added; 16 DSA is preferentially incorporated into the membrane and no bound probe can be detected in the low ionic strength extract of ghosts. To study the effects of different Abs, the spin label was added after ghost resealing. In contrast spin labeling with 4MSL was performed before ghost resealing at 0°C in 5 mM phosphate buffer pH 7.5. Membrane proteins (3 mg) were labeled with 0.1 mg of 4MSL. Excess of spin label was removed with three washes in the same buffer and ghosts were resealed. Care was observed to keep buffer pH, ionic strength, and spin label concentration constant in each experiment. These variables need to be controlled to give reproducible EPR spectra [16]. Low ionic strength extract of 4MSL-labeled ghosts and spectrin purification was performed according to Litman et al [17]. Spin labeling of low ionic strength extracted proteins was also performed after the extraction without any observable difference in the EPR spectra. Spectra were recorded on a Varian E-4 spectrometer (Palo Alto, CA) equipped with a variable temperature accessory. Temperature was monitored by a digital thermometer set above the cavity. We analyzed 16 DSA spectra by using the empirical parameter log ($h_0/h_{-1}$) (see [11] for the discussion on this parameter). At each temperature four spectra were recorded for 16 DSA spin label and the average value of peak heights used for calculation. Computer analysis of data has been performed as described [11]; 4MSL alkylates mainly the sulfhydryl groups of proteins and nonspecific incorporation in the lipid phase does not occur. The spectrum of 4MSL was analyzed by measuring the amplitude ratio of weakly (w) and strongly (s) immobilized components [16].

**Circular dichroism.** Circular dichroism measurements were made with a Jasco J-41 A spectrometer (Tokyo, Japan). Ellipticity in the region of peptide cotton effect was measured by using thermostated cells of 10-mm or 1-mm path-length. Heating rate was 0.33°C/min and controlled by a temperature programmer Haake PG-20 (Berlin, Germany).

**Immunological detection of proteins on nitrocellulose.** Specificity of monoclonal and polyclonal Abs was tested on RBC membrane proteins transferred to nitrocellulose sheets after sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Towbin et al [18]; $^{125}I$ protein A was employed to detect Abs binding. The nitrocellulose transfer was dried and subjected to autoradiography at −80°C with an intensifying screen for 1–3 days.

## RESULTS

### Identification of Proteins Involved in the 40°C Transition of RBC

16 DSA is a freely diffusible lipid spin probe used to explore the hydrophobic membrane core. A measurement of its degree of motion as a function of temperature in RBC membranes reveals the presence of multiple discontinuities possibly reflecting structural membrane transitions [11]. A plot of the empirical parameter log ($h_0/h_{-1}$)

vs the reciprocal of the temperature detects three transitions at 8°, 20°, and 40° ± 1.5°C (Fig. 1). In a previous work we observed that the selective extraction of skeletal proteins from RBC membrane abolished the 40°C transition [11]. To further characterize this transition we studied the effects of Abs specific for spectrin, protein 4.1, and for the cytoplasmic domain of band 3.

As shown in Figure 1, polyclonal affinity-purified antispectrin Abs added to ghosts at 1:1 molar ratio with respect to spectrin completely eliminate the 40°C transition. The transition at 8°C is virtually unmodified whereas the 20°C transition

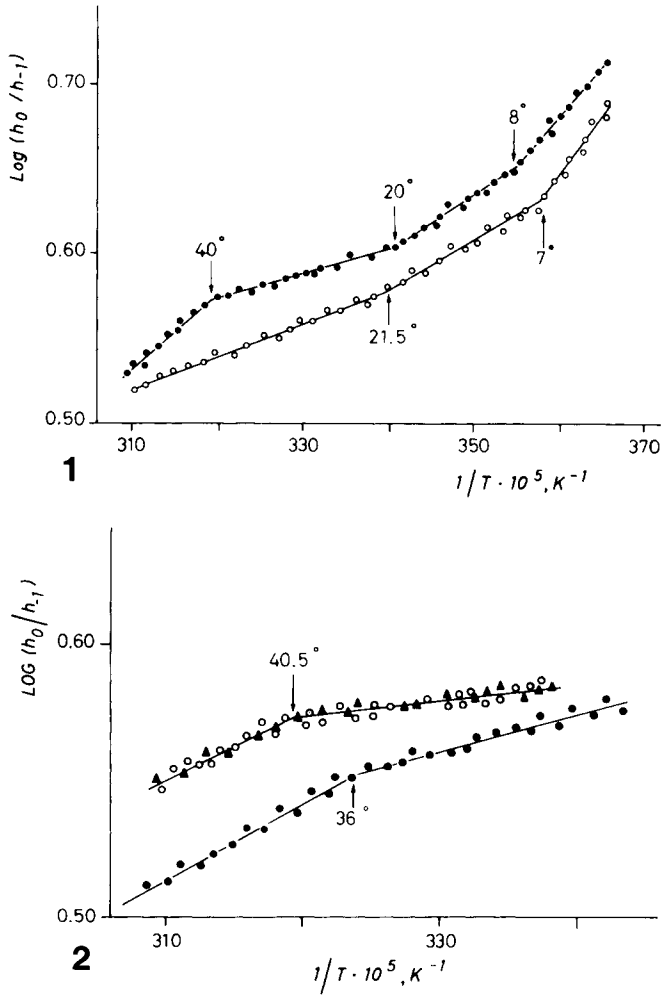



Fig. 1. Effects of polyclonal antispectrin Abs on the plot of log ($h_0/h_{-1}$) vs 1/T for 16 DSA-labeled ghosts. (●—●), control ghosts; (○—○), antispectrin Abs; spectrin:Abs molar ratio 1:1. Break temperatures were determined by computer analysis.

Fig. 2. Effects of polyclonal anti-4.1 protein and anti-band 3 cytoplasmic domain Abs on the plot of log ($h_0/h_{-1}$) vs 1/T for 16 DSA-labeled ghosts. (○—○), control ghosts; (▲—▲), anti-band 3 Abs; band 3:Abs molar ratio 1:3; (●—●), anti-4.1 protein Abs; protein 4.1:Abs molar ratio 1:1. Break temperatures were determined by computer analysis.

is less evident. Control experiments with the IgG fraction of the pre-immune sera did not show changes in the thermotropic breaks, thus excluding unspecific effects of IgG.

Figure 2 shows the results obtained with ghosts treated with polyclonal affinity-purified anti-4.1 protein Abs. At 1:1 molar ratio with respect to protein 4.1, these Abs produce a lowering of the transition temperature from 40°C to 36°C. In contrast, polyclonal affinity-purified Abs specific for the 43K cytoplasmic domain of band 3 did not affect the 40°C transition even when a 3:1 molar excess of anti-band 3 Abs with respect to the protein was used (Fig. 2).

## Thermotropic Properties of Spectrin

One possibility to explain the transition detected by 16 DSA at 40°C is that the involved protein (spectrin) undergoes a conformational change at this temperature. Several physicochemical techniques, including circular dichroism and calorimetry [4], fluorescence polarization [19], and spin labeling methods [20] detect a thermotropic unfolding of spectrin with an onset temperature of 38–45°C.

As previously reported by Brandts et al [4], we observed that circular dichroism (CD) spectra of purified spectrin at 222 nm, which reflect the backbone helical conformation, show a 10% reproducible decrease in the total ellipticity between 30 and 48°C (Fig. 3). This unfolding can be tentatively attributed to dimer-oligomer equilibrium, which is a temperature-dependent process, and partially to the dissociation of the two spectrin subunits that, as suggested by Calvert and Gratzer [21], precedes massive unfolding. Above 48°C a massive irreversible thermal unfolding of the protein occurs (Fig. 3).

Antispectrin Abs added at 1:1 molar ratio (at this concentration poly- and monoclonal Abs do not contribute to spectrin CD spectra) do not produce detectable

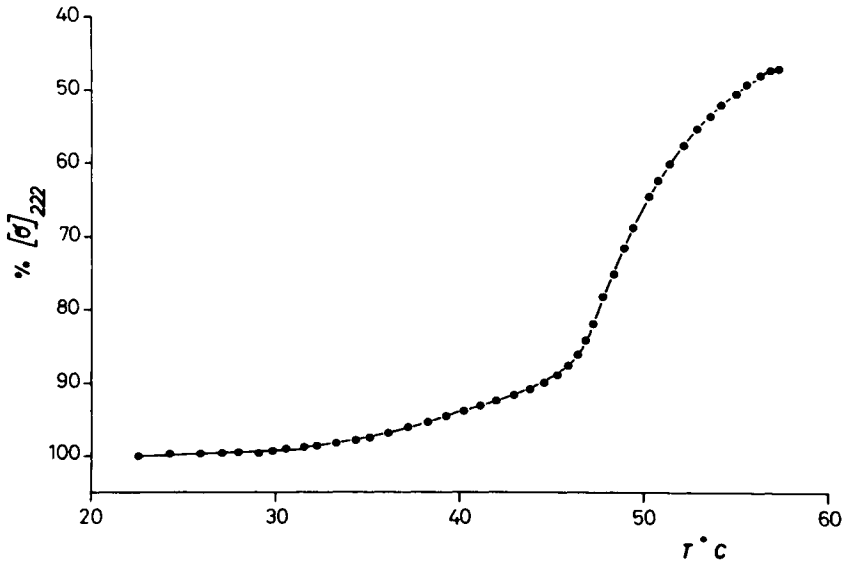



Fig. 3. Effect of temperature on circular dichroism spectra of purified spectrin at 222 nm. $\theta_{222}$ = percentage of residual ellipticity at 222 nm. Heating rate: 0.33°C/min.

modifications of spectrin thermal properties as measured by CD. Clearly this technique detects spectrin thermotropic properties arising from a multiple $\alpha$-helix domain (70–80% of this protein is in the $\alpha$-helix configuration [21]) and the binding of only one domain per spectrin molecule by Abs is not sufficient to produce observable effects.

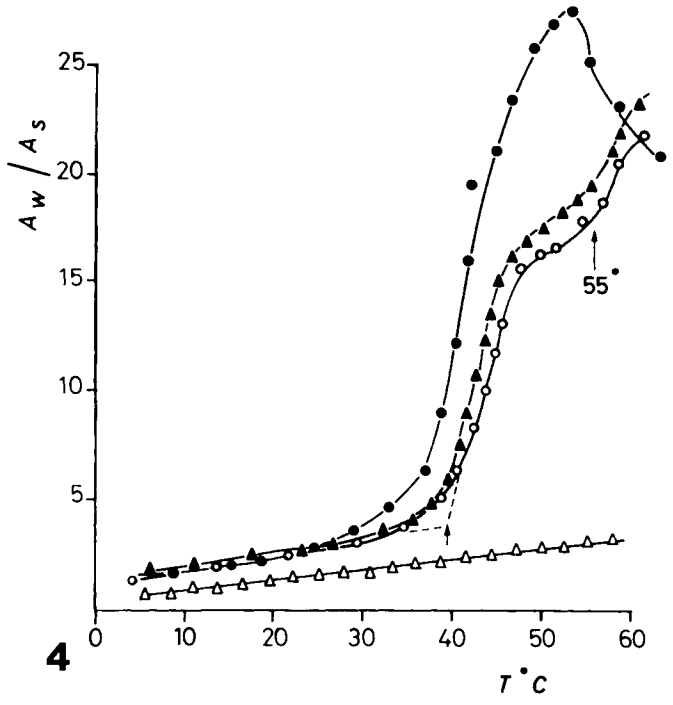
The use of maleimide spin labels to study physicochemical properties of RBC membrane proteins has been extensively discussed [22]. The maleimide spin label utilized in this work, 4MSL, detects a spectrin-dependent thermotropic transition in ghosts [22] that, unlike CD, derived from fixed protein sites neighboring $-$SH groups labeled by the probe. It is likely that the spectrin-actin complex accounts for approximately 80% of the signal of the 4MSL ghosts [22]. The results obtained with 4MSL membranes before and after the extraction of extrinsic proteins at low ionic strength are shown in Figure 4. Two transitions, with onset temperatures of 40 and 55°C, are detected in 4MSL ghosts. 4MSL-labeled inside-out vesicles do not show transitions, whereas both the thermotropic breaks are found in the low ionic strength extract (Fig. 4). This extract contains mainly spectrin and actin along with a small amount of protein 4.1. After 4MSL-spectrin purification on a Sepharose 4B column the presence of the 40°C transition is observed (Fig. 4).

The involvement of spectrin in the 40°C transition was further confirmed by using monospecific polyclonal antispectrin Abs. At 1:1 molar ratio these Abs inhibit the 40°C transition of 4MSL ghosts, and at higher concentrations a disappearance of the transition is observed (Fig. 5). As expected none of the Abs specific for other RBC proteins we used were able to affect 4MSL transition (results not shown). To study the effects of antispectrin Abs in more detail we utilized three monoclonal Abs as site-specific modifiers of spectrin structure in situ. One of the monoclonal Abs is specific for the 80K aminoterminal domain [14] of $\alpha$-spectrin subunit (hereafter referred to as anti-80K). The second monoclonal has been obtained from a mouse with an autoimmune disease producing Abs against its own spectrin (hereafter referred to as auto-I). This Abs reacts with internal domains of both $\alpha$- and $\beta$-subunits [Heltianu, Bologna, Speicher, Murphy, and Marchesi, unpublished observations]. The third monoclonal Abs is specific for the $\beta_I$ domain of spectrin (hereafter referred to as anti-$\beta_I$) [23].

The effects of these monoclonal Abs on 4MSL ghosts are shown in Figures 6 and 7. Both anti-80K and anti-$\beta_I$ Abs either at 1:1 molar ratio with respect to spectrin or at higher concentrations were ineffective in the inhibition of 40°C transition (Fig. 6). In contrast, auto-I Abs at a 1:1 molar ratio completely inhibit the transition (Fig. 7). Figure 7 shows that a measurable inhibition is also found with a 1:10 molar defect of Abs. This relatively strong effect is due to the sensitivity of the $A_w/A_s$ ratio. Although the $A_w$ peak is always higher than $A_s$, at room temperature the spectrum consists of about 10% of the signal in the weak (w) and 90% in the strong (s) state [22]. Therefore, a small change in the states will give a remarkable effect in the $A_w/A_s$ value, and the binding of monoclonal Abs to few spectrin domains containing the spin label is sufficient to produce an observable effect. On the other hand a preferential interaction of auto-I Abs with protein sites more accessible to the antibody for probe-induced local unfolding is also plausible.

## DISCUSSION

This study provides new evidence in favor of a role of proteins in RBC structural thermotropic transitions. Spectrin appears to be directly involved in both the 40°C



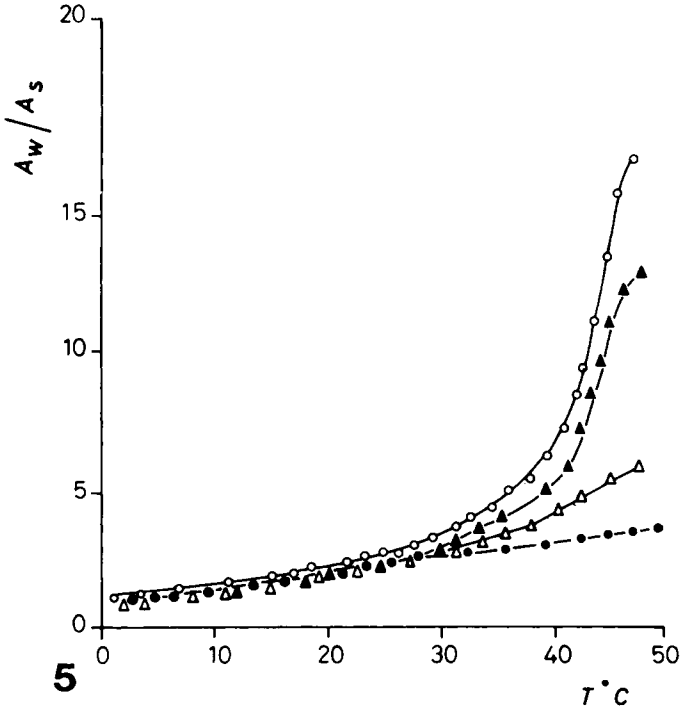




Fig. 4. Effect of temperature on $A_w/A_s$ ratio for 4MSL-labeled RBC membrane proteins. (○—○), control ghosts; (▲—▲), low ionic strength extract; (△—△), inside-out vesicles; (●—●), purified spectrin.

Fig. 5. Effects of polyclonal antispectrin Abs on $A_w/A_s$ ratio as a function of temperature for 4MSL-labeled ghosts. (○—○), control ghosts; antispectrin Abs at different molar ratios: (▲—▲), spectrin:Abs molar ratio 1:1; (△—△), spectrin:Abs molar ratio 1:4; (●—●), spectrin:Abs molar ratio 1:10.

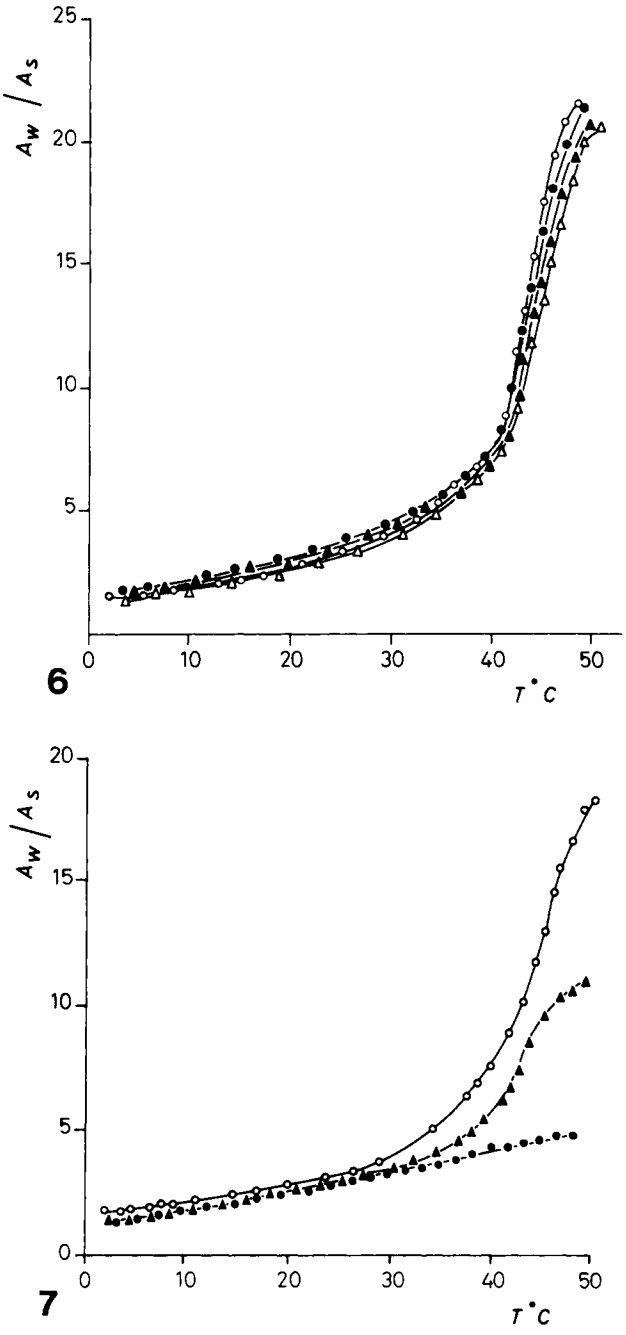



Fig. 6. Effects of monoclonal anti-$\beta_I$ and anti-80K antispectrin Abs on $A_w/A_s$ ratio as a function of temperature for 4MSL-labeled ghosts. (●—●), control ghosts; (○—○), anti-$\beta_I$ Abs; spectrin:Abs molar ratio 1:1; (△—△), anti-80K Abs; spectrin:Abs molar ratio 1:1; (▲—▲), anti-80K Abs; spectrin:Abs molar ratio 1:10.

Fig. 7. Effects of monoclonal auto-I antispectrin Abs on $A_w/A_s$ ratio as a function of temperature for 4MSL-labeled ghosts. (○—○), control ghosts; (●—●), spectrin:Abs molar ratio 1:1; (▲—▲), spectrin:Abs molar ratio 10:1.

break detected as a change in the freedom of motion of lipid spin probe 16 DSA and in the protein conformational change detected in 4MSL ghosts above 40°C. This interpretation is consistent with the fact that inside-out vesicles do not show the transitions detected by both the spin labels (Fig. 4) [11]. As is known, these membranes maintain the major intrinsic proteins of RBC ghosts but lack the spectrin-actin complex. We have shown in this study that antispectrin Abs are especially active as inhibitors of 40°C transition (Figs. 1, 5, 7). A further indication for a role of spectrin in the 40°C transition comes from its characteristic thermal behavior. Spectrin is a protein with high segmental mobility [21] and $\alpha$-helix melting profiles in low concentrations of urea or with elevated temperature suggest the presence of multiple labile structures even in solvent conditions that preserve native structure (Fig. 3) [21]. The decrease of ellipticity at 222 nm with temperature increasing indicates a continuous unfolding of the molecule starting around 30°C that, at 48°C, accounts for 10% of the total ellipticity (Fig. 3). Above 48°C, a massive and irreversible unfolding of spectrin is observed involving, at the end of the transition, nearly half of the total ellipticity (Fig. 3).

If spectrin unfolding is somehow reflected in the properties of the lipid bilayer, that would supply a possible mechanism to explain the modification of RBC physicochemical properties at the membrane level, as detected by 16 DSA. It is conceivable that spectrin unfolding modifies its interaction with the membrane. Previous workers have suggested that skeletal proteins can control the freedom of motion of both band 3 and glycophorin [9–10]. We have shown that Abs specific for protein 4.1 produce a lowering of the transition temperature. Recent studies suggest a key role of this protein in the assembly of RBC cytoskeleton. Protein 4.1 not only appears to modulate spectrin-actin association [24], but also interacts strongly with glycophorin [25]. This membrane binding involves specific phospholipids and could be relevant for skeleton-membrane interactions and other functions [26, 27]. Our data suggest that protein 4.1 may be involved in some way in the transfer to the membrane of spectrin thermal changes. The binding of anti-4.1 protein Abs might produce a weakening of one of the spectrin attachment sites, and this could be reflected in a lowering of the transition temperature. The cytoplasmic domain of band 3 is known to bind spectrin through 2.1 protein (ankyrin) [28]; even though there is about an eight to ten molar excess of band 3 to 2.1 protein, we observed that anti-band 3 Abs did not modify the 40°C transition. Thus, at least the band 3 domains recognized by our polyclonal Abs do not seem to be directly involved in the transition.

We investigated spectrin thermotropic properties with 4MSL in situ and after extraction and purification. The question raised by a comparison between EPR and CD studies of spectrin is whether the difference of transition temperatures (ie, 40°C for EPR and 48°C for CD) could be due to probe-induced perturbations; 4MSL could unfold spectrin molecules near the labeled −SH groups if the spin label causes the exposure of otherwise buried internal domains. In this way, the spin label could detect conformational changes of spectrin occurring at a temperature lower than that which induces massive protein unfolding. Similar arguments can be considered at the membrane level for the transition temperature detected by 16 DSA. We have no experimental way of discriminating between these two possibilities at the present time. However, 4MSL-labeled sites appear to be different with respect to the majority of −SH groups and it is likely that not all the labeled groups participate in the transition (see below). Moreover, fluorescence polarization studies of spectrin intrin-

sic tryptophans showed cooperative thermal changes starting above 38°C [19]. The hypothesis that different spectroscopic techniques detect thermotropic properties of different folding domains seems to be plausible.

We observed that a 1:1 molar ratio of auto-I Abs is sufficient to completely abolish the 40°C transition of 4MSL ghosts. This result indicates that only a few −SH groups are involved in the transition. Thus, labeled groups appear not to be equivalent from an immunological and thermotropic point of view.

The other monoclonal Abs tested have been found ineffective in the inhibition of 40°C transition of 4MSL ghosts, thus suggesting that the domains recognized by these Abs do not contain the −SH groups labeled by the spin probe.

These studies should facilitate the characterization of functional and topological aspects of the RBC skeleton, and the identification of membrane components involved in thermal properties of RBC. The knowledge of labeling sites should provide a powerful approach to the elucidation of molecular defects in pathological membranes.

## ACKNOWLEDGMENTS

We are indebted to Dr. Paola De Cesaris for her assistance and collaboration and to Giuseppe Scorza and Stefano Barca for their excellent technical assistance.

This research was partially supported by NATO research grant No. 236/84 and by a grant of the Italian National Research Council, Special Project "Oncology" contract No. 84.00730.44.

## REFERENCES

1. Galla HJ, Luisetti J: Biochim Biophys Acta 596:108–117, 1980.
2. Nigg EA, Cherry RJ: Biochemistry 18:3457–3465, 1979.
3. Minetti M, Ceccarini M: J Cell Biochem 19:59–75, 1982.
4. Brandts JF, Erickson L, Lysko K, Schwartz AT, Taverna RD: Biochemistry 16:3450–3454, 1977.
5. Hui SW, Stewart CM, Carpenter MP, Stewart TP: J Cell Biol 85:283–291, 1980.
6. Verma SP, Wallach DFH: Proc Natl Acad Sci USA 73:3558–3561, 1976.
7. Marchesi VT: Blood 61:1–11, 1983.
8. Cherry RJ: Biochim Biophys Acta 559:289–327, 1979.
9. Golan DE, Veatch W: Proc Natl Acad Sci USA 77:2537–2541, 1980.
10. Butterfield DA, Bennett TF, Feix JB: Ann NY Acad Sci 414:169–179, 1983.
11. Minetti M, Ceccarini M, Di Stasi AMM: J Cell Biochem 25:73–86, 1984.
12. Goodman SR, Shiffer K: Am J Physiol 244:C121–C141, 1983.
13. Leto TL, Marchesi VT: J Biol Chem 259:4603–4608, 1984.
14. Yurchenko PD, Speiker DW, Morrow JS, Knowles WJ, Marchesi VT: J Biol Chem 257:9102–9107, 1982.
15. Nicolson GL, Painter RG: J Cell Biol 59:395–406, 1973.
16. Fung LW-M, Simpson MJ: FEBS Lett 108:269–273, 1979.
17. Litman D, Hsu CJ, Marchesi VT: J Cell Sci 42:1–22, 1980.
18. Towbin H, Staehelin T, Gordon J: Proc Natl Acad Sci USA 76:4350–4354, 1979.
19. Yoshino H, Marchesi VT: J Biol Chem 259:4496–4500, 1984.
20. Cassoly R, Daveloose D, Leterrier F: Biochim Biophys Acta 601:478–489, 1980.
21. Calvert R, Ungewickell E, Gratzer W: Eur J Biochem 107:363–367, 1980.
22. Fung LW-M: Ann NY Acad Sci 414:162–168, 1983.
23. Marchesi VT: "Erythrocyte Membrane 3. Recent Clinical and Experimental Advances." New York: Alan R. Liss, pp 1–12, 1984.
24. Ungewickell E, Bennett PM, Calvert R, Ohanian V, Gratzer WG: Nature 280:811–814, 1979.
25. Anderson RA, Lovrien RE: Nature 307:655–658, 1984.
26. Anderson RA, Marchesi VT: J Cell Biol 97:297 (Abstr.), 1983.
27. Pasternak G, Leto TL, Anderson R, Bottomly K, Marchesi VT: J Cell Biol 99:1111 (Abstr.), 1984.
28. Bennett V, Stenbuck PJ: J Biol Chem 255:6424–6432, 1980.