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## Protein 4.1 Is Involved in a Structural Thermotropic Transition of the Red Blood Cell Membrane Detected by a Spin-Labeled Stearic Acid<sup>†</sup>

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**ABSTRACT:** Proteins involved in a structural transition in red blood cell membranes detected at  $8 \pm 1.5$  °C by a stearic acid spin-label have been investigated. Calcium loading of red blood cells with ionophore A23187 caused the disappearance of the 8 °C transition. Protein 4.1 appears to be the most susceptible protein to  $\text{Ca}^{2+}$  treatment. Antibodies specific for spectrin, band 3 (43K cytoplasmic domain), and protein 4.1 have been utilized as specific probes to modify membrane thermotropic properties. The 8 °C transition was eliminated by anti-4.1 protein antibodies but was not modified by the other antibodies. To further characterize the protein(s) involved in the transition, ghosts were subjected to sequential extraction of skeletal proteins. The extraction of band 6, spectrin, and actin did not modify the 8 °C transition. In contrast, high-salt extraction (1 M KCl) of spectrin-actin-depleted vesicles, a procedure that extracts proteins 2.1 and 4.1, was able to eliminate the 8 °C transition. Rebinding of purified protein 4.1 to the high salt extracted vesicles restored the 8 °C transition. These results indicate the involvement of protein 4.1 in the transition and suggest a functional membrane association of this protein. The binding of protein 4.1 to the membrane seems to contribute significantly to the thermotropic properties of red blood cells.

The occurrence of structural transitions in plasma membranes of prokaryotic and eukaryotic cells has been described by several groups (Linden et al., 1973; Inesi et al., 1973; Wisnieski et al., 1974; Wetton et al., 1983). Structural changes involved in these transitions, especially for plasma membranes of mammalian cells, are not well understood due to the high level of complexity (reflected in a high level of anisotropy) of these membranes.

In the case of the red blood cell (RBC)<sup>1</sup> membrane, the occurrence of structural transitions in the 0-50 °C range has been reported by a variety of physicochemical techniques (Verma & Wallach, 1976; Nigg & Cherry, 1979; Hui et al., 1980; Galla & Luisetti, 1980; Minetti et al., 1984a). Structural changes involved in these transitions are still unclear, but it is interesting to note that the lateral mobility of glycoproteins changes discontinuously with temperature (Nigg & Cherry,

1979) and appears to be controlled by skeletal proteins (Golan & Veatch, 1980; Schindler et al., 1980).

In a previous work we reported evidence in favor of a major role of proteins in these transitions (Minetti et al., 1984a). The identification of membrane proteins involved in the observed thermotropic changes may be useful to understand temperature-dependent membrane phenomena. Temperature affects not only membrane enzymatic activities (Ogiso et al., 1981) but also resealing and hemolysis processes. A common critical temperature for both lysis and resealing processes appears to be at 7-10 °C (Minetti & Ceccarini, 1982; Minetti et al., 1984b). The lipophylic stearic acid 16-nitroxide (16-NS) spin-label inserted into intact RBC exhibited a significant change in its degree of motion at  $8 \pm 1.5$  °C. Treatments that inhibit cold-induced hypertonic hemolysis and decrease the

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<sup>1</sup> Abbreviations: RBC, red blood cell; 16-NS, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyl-1-oxy; Abs, antibodies; DFP, diisopropyl fluorophosphate; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

temperature dependence of hypotonic hemolysis caused a disappearance of the 8 °C transition (Minetti et al., 1984b).

In this work we have used selective protein extractions and specific antibodies (Abs) to identify RBC membrane protein(s) possibly involved in the 8 °C transition. Our data indicate a direct involvement of protein 4.1 in this transition [nomenclature of protein according to Steck (1974)]. We suggest that the interactions of this protein with the membrane contribute significantly to the thermotropic properties of RBC.

#### EXPERIMENTAL PROCEDURES

**Materials.** Stearic acid 16-nitroxide spin-label (16-NS) was obtained from Syva (Palo Alto, CA). DFP was obtained from Fluka (Buchs, Switzerland) and PMSF from Sigma (St. Louis, MO).

**Membrane Preparations and Rebinding Studies.** Unsealed ghosts were prepared from fresh human RBC with 5 mM sodium phosphate pH 8 buffer, at 0 °C. Sequential extraction of peripheral proteins was performed following known procedures. Briefly, band 6 was extracted by 0.6 M KCl before spectrin extraction (Fairbanks et al., 1971). Spectrin and actin were extracted at 37 °C for 30 min by 0.3 mM sodium phosphate, 0.1 mM EDTA, and 0.2 mM DFP (Morrow & Marchesi, 1981). Proteins 2.1 and 4.1 were removed by extraction of spectrin-actin-depleted vesicles with 1 M KCl, 1 mM EGTA, and 0.1 mM PMSF, pH 7.8. Protein 4.1 was purified by DEAE-cellulose chromatography according to Tyler et al., (1979) and concentrated by dialysis against poly(ethylene glycol) (Aquacide). For rebinding experiments, 1 mg of purified protein 4.1 was incubated with vesicles depleted of peripheral proteins (2 mL) and dialyzed against 130 mM KCl–5 mM sodium phosphate, pH 8 buffer for 2 h at 4 °C.

**Calcium-Ionophore Treatment.** A 0.5-mL sample of washed RBC was incubated for 20 min at 37 °C with 5 mL of 5 mM Tris, 40  $\mu$ M ionophore A23187, and 1.2 mM  $\text{CaCl}_2$  pH 8 buffer. RBC were washed with 5 mM Tris, 0.145 M NaCl, and 5 mM EGTA pH 8 buffer and then lysed at 0 °C. Membrane proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described by Laemmli (1970). Gels were stained with Coomassie Blue, and electrophoretic profiles were recorded with a Zeiss chromatographic spectrometer PMQ 3 (Zeiss, Oberkochen, West Germany).

**Reaction with Antibodies.** Polyclonal affinity-purified antibodies monospecific for spectrin, the cytoplasmic domain of band 3, and protein 4.1 were obtained and purified by standard techniques, and their specificity was demonstrated by immunoblot analysis of both intact proteins and specific chemical domains prepared by controlled proteolytic digestion (Pasternack et al., 1985; Leto & Marchesi, 1984). The amount of Abs was calculated on the basis of protein data reported by Branton et al. (1981). Abs were dissolved at 0.5–1 mg/mL in 5 mM sodium phosphate pH 8 buffer and added to unsealed ghosts at 0 °C. After 10 min of incubation at the same temperature, to allow Abs diffusion inside the membrane, ghosts were resealed at 37 °C with a buffer containing 150 mM KCl, 5 mM sodium phosphate, 0.25 mM ATP, 0.25 mM  $\text{MgCl}_2$ , 1 mM DFP, and 0.1 mM PMSF, pH 7.2. Membranes were checked for permeability to macromolecules before and after the resealing with fluorescent dextrans as previously described (Minetti & Ceccarini, 1982).

**Instrumentation.** Spectra were recorded on a Varian E-4 spectrometer (Varian Associates, Inc., Palo Alto, CA) operated at 9.5 GHz, 3261-G field set, 100-kHz field modulation, 1.25-G peak to peak modulation amplitude, and 18-mW microwave and equipped with a variable-temperature acces-

sory. Temperature was monitored by a digital thermometer set above the cavity. After an equilibration time of 2 min, the measured temperature gradient at the beginning and at the end of the sample did not exceed  $0.5 \pm 0.1$  °C in the 0–50 °C temperature range.

**Spin-Labeling and Spectra Analysis.** Before spin-labeling, intact RBC and resealed ghosts were washed by centrifugation with 150 mM NaCl and 5 mM sodium phosphate, pH 7.2, and inside-out vesicles were washed with 120 mM KCl, 20 mM NaCl, and 5 mM sodium phosphate, pH 7.2 buffers. The 16-NS spin probe was dissolved in ethanol at the concentration of 10 mM. To 1.8  $\mu$ g of probe, evaporated under a nitrogen stream, an amount of pelleted membranes corresponding to 0.3 mg of lipids was added. The spin-label incorporation was performed by overnight incubation at 0 °C in the dark and under a nitrogen atmosphere. The sample was then injected into a sealed capillary 4 cm long (50- $\mu$ L micropet, Clay Adams Div., Parsippany, NJ), and the capillary was set into the quartz tube. Although the exact localization of 16-NS into biological membranes is unknown, we always found the probe associated with membranes (only a negligible amount of free spin-label is superimposed to the membrane-bound label, see the insert of Figure 2). Moreover, no bound probe has been found in both the high and low ionic strength extracts of membrane proteins. Changes in the freedom of motion of 16-NS with temperature were analyzed with the empirical parameter  $\log(h_0/h_{-1})$ . Determination of this motional parameter is described elsewhere (Minetti et al., 1984a). At each temperature four spectra were recorded, and the average value of peak heights was used for computer analysis (Minetti et al., 1984a).

#### RESULTS

**Ca-Ionophore Treatment of RBC.** Erythrocytes treatment with the Ca ionophore A23187 in the presence of 1.2 mM external calcium results in a selective proteolysis of some skeletal proteins (Siegel et al., 1980), in the cross-linking of membrane proteins (King & Morrison, 1977; Seifring et al., 1978), and in an increased content of phosphatidylethanolamine and phosphatidylserine in the outer membrane leaflet (Schwartz et al., 1985).

As shown in Figure 1, one of the membrane proteins most affected by  $\text{Ca}^{2+}$  treatment appears to be band 4.1, which disappears after 20 min of ionophore treatment. Moreover, as a consequence of  $\text{Ca}^{2+}$  treatment, high molecular weight complexes are seen on the top of the gel, and some loss of band 3 is also evident (Figure 1). The effects of  $\text{Ca}^{2+}$  treatment on the thermotropic properties of RBC are shown in Figure 2. The disappearance of the low temperature break and the appearance of two breaks at 32 °C and 15 °C are observed (Figure 2). The 15 °C transition could be attributed to a lowering of the 20 °C transition. We have previously shown that the lipid-dependent 20 °C transition of RBC can be lowered to 15 °C by proteolytic treatments of unsealed ghosts (Minetti et al., 1984a). The residual transition at 15 °C is insensitive to further proteolysis, thus allowing us to distinguish this transition from that at 8 °C. The lowering effect on the 20 °C transition of  $\text{Ca}^{2+}$ -treated RBC could be due to the above-mentioned calcium-induced membrane modifications (i.e., proteolysis, protein cross-linking, aminophospholipid reorientation). The transition that occurs at 40 °C in control RBC has been attributed to spectrin (Minetti et al., 1986) and, after  $\text{Ca}^{2+}$  treatment, is lowered to 32 °C (Figure 2).

**Effects of Antibodies on RBC Thermotropic Properties.** To identify the protein(s) involved in the 8 °C transition detected by 16-NS, we studied the effects of Abs specific for spectrin, the cytoplasmic domain of band 3, and protein 4.1. Abs were

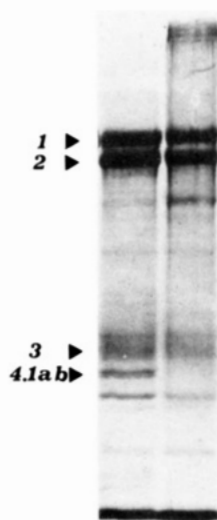


FIGURE 1: Effects of  $\text{Ca}^{2+}$  treatment on the electrophoretic pattern of RBC ghosts. Experimental conditions were as described under Experimental Procedures. A total of 60  $\mu\text{g}$  of control (left) and A23187-treated (right) ghosts was loaded on 6% sodium dodecyl sulfate-polyacrylamide gels and stained with Coomassie blue.

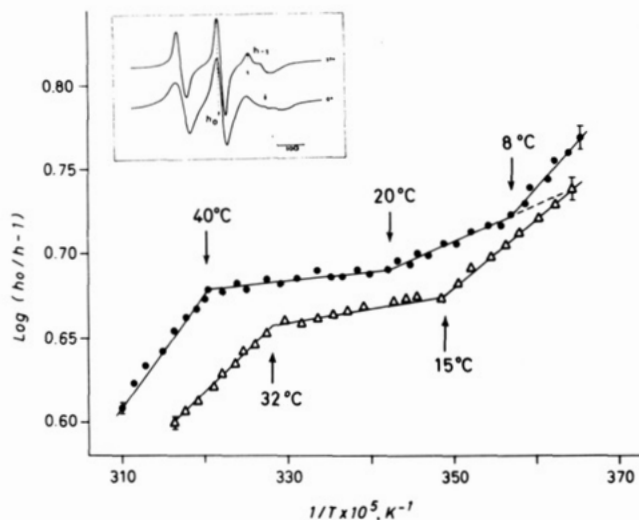


FIGURE 2: Effects of  $\text{Ca}^{2+}$  treatment on the thermotropic behavior of 16-NS in intact RBC: (●) control RBC; (Δ) Ca-A23187-treated RBC. The insert shows typical spectra at 0 and 37 °C of 16-NS in intact RBC; the arrow indicates the position of free spin-label. Determination of the motional parameter,  $\log(h_0/h_{-1})$ , is described elsewhere (Minetti et al., 1984a). Points are average values of four spectra; experimental errors usually decrease with increasing temperature; typical values  $\pm$  SEM are indicated. Break temperatures were determined by computer analysis.

added to ghosts at a 1:1 molar ratio with respect to the specific protein. The binding of Abs is believed to modify the conformation and/or the membrane binding of these proteins and thereby affects the RBC thermotropic properties.

As shown in Figure 3, anti-protein 4.1 Abs treatment completely eliminates the 8 °C transition. Ghosts resealed with anti-spectrin and anti-band 3 Abs do not affect the transitions and show two transitions at 20 and 8 °C identical with that seen with control ghosts (Figure 3).

**Effects of Selective Protein Extractions on RBC Thermotropic Properties.** To further characterize proteins involved in the 8 °C transition, RBC were subjected to sequential selective extractions of membrane proteins. Figure 4 shows that both the high-salt treatment (0.6 M KCl) of unsealed ghosts (this treatment removes band 6) and the low ionic

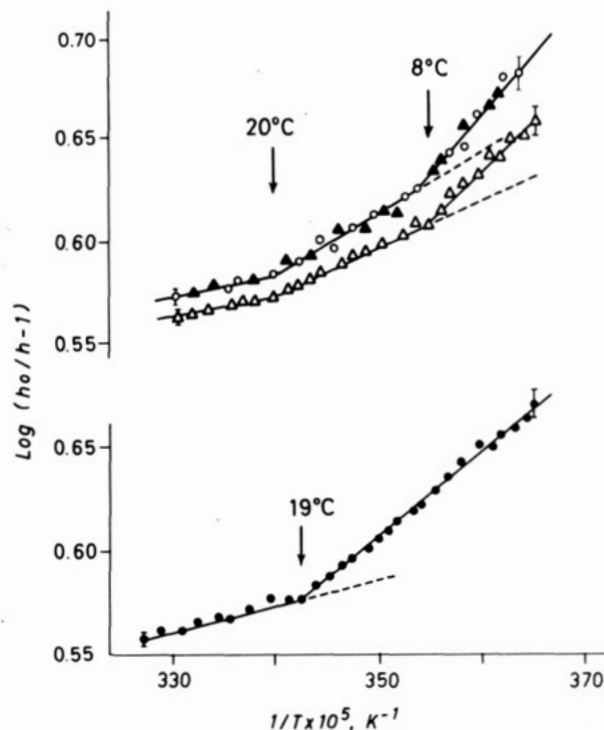


FIGURE 3: Effects of monospecific affinity-purified antibodies on the thermotropic behavior of 16-NS in resealed ghosts: (Δ) ghosts resealed with IgG of the preimmune sera; (▲) ghosts resealed with anti-spectrin Abs; (○) ghosts resealed with Abs specific for band 3 cytoplasmic domain; (●) ghosts resealed with anti-4.1 Abs. Data analysis was as in Figure 2.

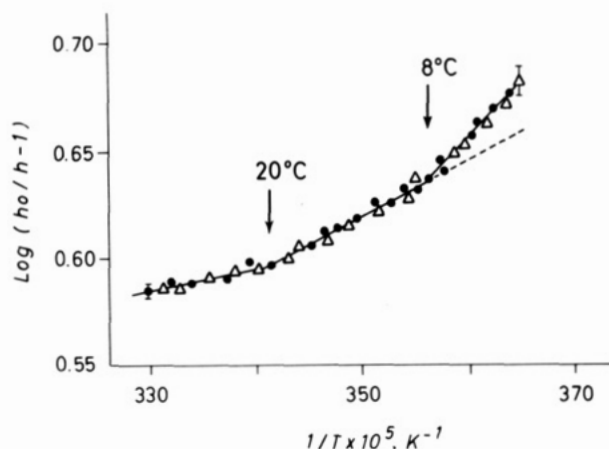


FIGURE 4: Effects of selective protein removal on the thermotropic behavior of membrane-bound 16-NS: (●) unsealed ghosts treated with 0.6 M KCl to extract band 6; (Δ) unsealed ghosts treated with 0.2 mM phosphate at 37 °C to extract spectrin and actin. Data analysis was as in Figure 2.

strength extraction of spectrin and actin did not affect the transitions at 8 and 20 °C. In contrast, the subsequent extraction of spectrin-actin-depleted vesicles with high salt (1 M KCl) abolished the 8 °C transition (Figure 5a). This last treatment removes proteins 2.1 and 4.1 from the membranes. The residual transition at 16 °C (Figure 5a), being protease insensitive, can be attributed to a lowering of the 20 °C transition and could be due to the extraction of peripheral membrane proteins or to proteolytic degradation of membrane proteins.

The effects of 1 M KCl on the thermotropic properties of spectrin-actin-depleted vesicles are not considered to be due to the high ionic strength; in fact, (i) the 8 °C transition was not restored after two washings with 0.15 M NaCl and 5 mM

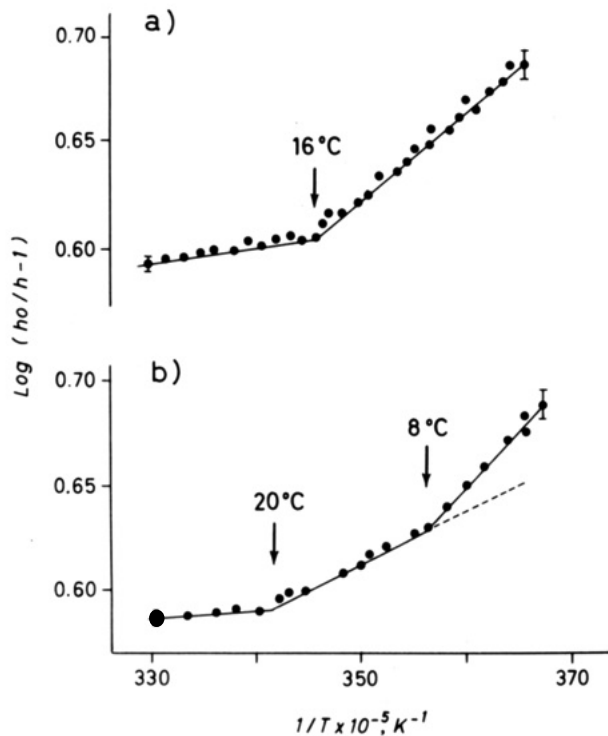


FIGURE 5: Effects of protein 4.1 extraction and protein 4.1 rebinding on the thermotropic behavior of 16-NS in vesicles stripped of peripheral proteins: (a) vesicles depleted of spectrin and actin after 1 M KCl treatment; (b) vesicles as in (a) after rebinding with purified protein 4.1. A total of 1 mg of protein 4.1 was incubated with 2 mL of vesicles depleted of peripheral proteins (see Experimental Procedures). Data analysis was as in Figure 2.

sodium phosphate, pH 7.5 buffer, and (ii) resealed ghosts treated with high salt without a previous extraction of spectrin and actin showed the 8 °C transition apparently unmodified (data not shown).

These results taken together with those obtained with anti-protein 4.1 Abs suggest the involvement of this protein in the 8 °C transition, and therefore, we studied the effects of protein 4.1 rebinding on vesicles depleted of extrinsic proteins. Protein 4.1 was purified by DEAE-chromatography (Tyler et al., 1979) and added to inside-out vesicles previously stripped of extrinsic proteins. The high-salt treatment of spectrin-actin-depleted vesicles does not exhaustively remove protein 4.1 (Figure 6). An evaluation of the band 3/band 4.1 ratio before and after high-salt treatment obtained by cutting and weighing the area of Coomassie blue peaks indicated a residual 30% of protein 4.1 remaining associated with vesicles after 1 M KCl extraction. After rebinding, an increased amount (185% beyond that normally present) of protein 4.1 has been found associated with these vesicles (Figure 6). This increased amount of membrane-bound protein 4.1 is presumably due to a second binding site to band 3 (Pasternack et al., 1985). However, the removal of 70% of protein 4.1 is sufficient to eliminate the 8 °C transition, and the transition is restored after rebinding to vesicles depleted of peripheral proteins of purified protein 4.1 (compare Figures 5b and 4).

# DISCUSSION

Data reported in this paper show that the use of the 16-NS spin-label to study the thermotropic properties of RBC provides a way to identify reproducible thermotropic breaks that can be selectively modified by specific Abs or by sequential protein extractions. Several lines of evidence suggest that protein 4.1 is involved in the 8 °C transition of RBC membrane.  $Ca^{2+}$  treatment, although producing several changes of membrane

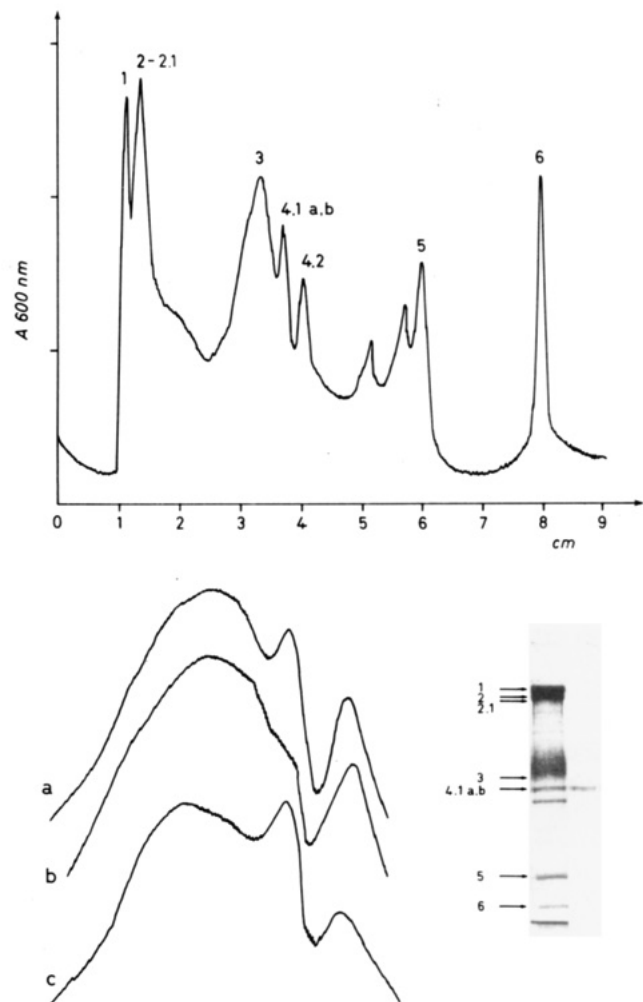


FIGURE 6: Electrophoretic profiles of membrane proteins in rebinding studies with purified protein 4.1: (upper) electrophoretic profiles of intact ghosts; (lower) band 3-band 4.2 zone of the gel (a) before protein 4.1 extraction, (b) after protein 4.1 extraction, and (c) after rebinding with purified protein 4.1. Rebinding conditions were as in Figure 5.

proteins (King & Morrison, 1977; Siegel et al., 1980) and lipids (Schwartz et al., 1985), results in the disappearance of both band 4.1 and the 8 °C transition (Figures 1 and 2). Antibodies against protein 4.1 are able to inhibit the 8 °C transition (Figure 3) while Abs against spectrin and the cytoplasmic domain of band 3 are not.

Although the high-salt treatment of spectrin-actin-depleted vesicles extracts both protein 2.1 (ankyrin) and protein 4.1, a significant role of ankyrin seems to be unlikely because (i) antibodies specific for the domain of band 3, which is responsible for ankyrin binding, do not affect the transition and (ii) the rebinding of purified protein 4.1 to membranes is sufficient to restore the transition.

The reversibility of the 8 °C transition is consistent with the postulated membrane interaction of protein 4.1 through a specific high-affinity protein-protein association (Anderson & Lovrien, 1984). Reassociation studies suggest a key role of protein 4.1 in both skeleton-membrane interactions and stabilization of a complex with spectrin and actin. The membrane interaction seems to involve glycoporphin A and polyphosphoinositides as lipid cofactors (Anderson & Lovrien, 1984; Anderson & Marchesi, 1983). In addition, protein 4.1 has also been reported to bind glycoporphin C (Mueller & Morrison, 1981) and phosphatidylserine (Sato & Ohnishi, 1983), and Pasternack et al. (1985) have shown that band 3

is a second class of integral membrane proteins to which protein 4.1 can bind. These skeleton-membrane complexes appear to be likely candidates for the membrane domains responsible for the 8 °C transition.

In a previous paper we reported that RBC spectrin could be involved in a structural membrane transition detected by 16-NS at 40 °C (Minetti et al., 1984). At this temperature spectrin undergoes a thermal unfolding that could be the origin of the transition detected by 16-NS at the membrane level, and we have found that anti-4.1 Abs are able to decrease the transition temperature from 40 to 35 °C (Minetti et al., 1986). In the present work we have shown that  $\text{Ca}^{2+}$  treatment affects band 4.1 and decreases the 40 °C transition to 32 °C (Figure 2).

A precise explanation of the physical changes responsible for the 8 °C transition is difficult at the present time since the membrane anisotropy hinders a precise estimate of 16-NS motions within biological membranes (Schreier et al., 1978). A change of membrane properties due to thermal unfolding of skeletal proteins, as we suggested for the 40 °C transition, appears to be unlikely, at 8 °C, and futher, protein 4.1 labeled with 4-maleimido-2,2,6,6-tetramethylpiperidiny-1-oxy spin-label did not show any thermal change in the 0–40 °C temperature range (M. Minetti and T. L. Leto, unpublished results). On the contrary, a protein-dependent lipid lateral phase separation could be a likely hypothesis in view of the postulated protein-lipid interactions of protein 4.1 (Anderson & Marchesi, 1983; Sato & Ohnishi, 1983). Consistent with this hypothesis, Raman spectroscopy appears to detect at 10 °C a transition arising from lipid or protein-lipid domains (Verma & Wallach, 1976; Verma & Wallach, 1982).

It should be emphasized that two of the three transitions detected by 16-NS in the RBC membrane depend on the presence of skeletal proteins. A role of membrane skeleton in RBC transitions could be relevant for the understanding of some temperature-dependent membrane phenomena. Ghost resealing starts above 10 °C (Minetti & Ceccarini, 1982), and below this critical temperature RBC can be easily lysed in both hypertonic and hypotonic media (Minetti et al., 1984b). We suggest that the membrane domains involved in hemolysis-resealing processes involve in some way the action of protein 4.1.

The identification of proteins involved in thermal properties of membranes, as attempted in this study, should be helpful to the RBC membrane topological and pathophysiological characterization.

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