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Properties of Red Cell Membrane Proteins: Mechanism of Spectrin and Band 4.1 Interaction†

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ABSTRACT: Interactions between human red cell's band 4.1 and spectrin were studied by fluorescence resonance energy transfer and batch microcalorimetry techniques. The association constant (K_a = 8.6 × 10^7 M^{-1}), the stoichiometry (one molecule of band 4.1 to one molecule of spectrin), the reversibility, and the enthalpy (ΔH = −6 kcal/mol) were determined. A proton uptake was observed to take place as a result of the spectrin–band 4.1 complex formation. In addition to the protonation of the reaction products, the entropic contribution ($-T\Delta S$) has been observed to be responsible for approximately 50% of the binding free energy. We concluded that the environment plays a significant role in the stabilization of the complex. Since band 4.1 has been required for the maintenance of the cytoskeletal stability, small alterations of the binding energies or the degree of interaction could have a pronounced effect on the structure of the erythrocyte membrane.

Although the molecular organization of the human red cell membrane has been a center of intensive research, several fundamental gaps in the understanding of what makes cells both elastic and durable still remain unfilled. In the process of studying structure and function of mammalian erythrocytes, it has become clear that the membranes' shape and mechanical properties are defined by a network of cytoplasmic proteins, named a cytoskeleton [see reviews by Steck (1974), Marchesi et al. (1976), Lux (1979), and Branton (1981)]. Since then, attempts to elucidate the structure of the erythrocyte mem-

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brane have focused on reassembly of the major cytoskeletal proteins in prepurified form in solutions. Spectrin, band 4.1, and actin, the major constituents of the cytoskeleton, were observed to form a cross-linked network (Ungewickell et al., 1979; Cohen et al., 1980; Pinder & Gratzer, 1983). Although Brenner & Korn (1979) have demonstrated by viscometry that spectrin tetramers can cross-link and cause gelation of F-actin in the absence of band 4.1, the spectrin–F-actin complex appears to be much stronger in the presence of band 4.1 (Ungewickell et al., 1979). Furthermore, the gels of spectrin and F-actin formed with band 4.1 are thixiotropic as judged by a fast reannealing ability subsequent to shearing (Cohen & Korsgren, 1980).

More recently, Elbaum et al. (1984) observed that the complex of spectrin and band 4.1, but neither spectrin nor band 4.1 alone, stimulated the rate of nucleation and stabilized oligomers of F-actin. Ohanian et al. (1984) analyzed the ternary interactions between the proteins and determined that the interactions between spectrin dimers and F-actin were increased by 9 orders of magnitude in the presence of band 4.1 as judged by the affinity constants.

Cohen & Foley (1984), in an attempt to determine the stoichiometry of spectrin, band 4.1, and actin complex formation, reported that the molar ratio of band 4.1 and spectrin was not fixed, ranging from 0.6 to 2.2. However, they concluded that a single molecule of band 4.1 was sufficient to promote the binding of spectrin dimer to F-actin.

In light of the fact that the only data available aimed to understand the energy of binding between spectrin, actin, and band 4.1 have been based on heterogeneous assays (requiring a phase separation of free from bound material) and obvious limitations of the above methodologies, we have attempted to determine the thermodynamics of interactions of the binary mixtures of dimeric spectrin with band 4.1 by techniques where the substrates and the products of the reaction were present in the same phase. Fluorescence energy transfer and microcalorimetry were employed to determine the stoichiometry, the free energy, and the enthalpy of the reaction. We observed that the formation of the spectrin dimer–band 4.1 complex results in an uptake of proton. This finding suggests that the environment controls the strength and the degree of not only the reaction but also the cytoskeleton as well.

Materials and Methods

Materials. Fluoresceinyl isothiocyanate I (FITC),[1] bovine serum albumin (BSA), DEAE-cellulose (mesh medium), and L-histidine were purchased from Sigma. Eosinyl-5-maleimide was a Molecular Probes product. Sepharose 2B was obtained from Pharmacia. All other common materials used for buffers were of at least reagent grade. No more than 1 week outdated blood was obtained from the Jacobi Hospital Blood Bank (Bronx, NY).

Preparation of Erythrocyte Ghosts. Ghosts were prepared from human blood by the method of Dodge et al. (1963). Packed red cells were resuspended in 10 volumes of 0.9% NaCl solution and centrifugated (5 min at 2000 rpm). Following three washes in this solution, the erythrocytes were lysed in 20 volumes of lysis buffer (5 mM sodium phosphate, 1 mM EDTA, pH 7.9) and immediately centrifuged (10 min at 2 °C, 9000 rpm). After removal of the button of granulocyte debris, the membrane pellet was resuspended in the above buffer and centrifuged once more. The ghosts were washed 5–7 times in a similar manner until white.

Preparation of Spectrin Dimer and Band 4.1. Spectrin dimer was prepared by previously described methods (Ralston 1976; Tyler et al., 1980). Spectrin dimer was extracted from membranes by incubation in 0.5 mM sodium phoshate buffer, pH 7.8, containing 0.1 mM EDTA, 0.1 mM NaCl, 0.2 mM DTT, and 2 mM NaN_3, at 37 °C for 30 min. The extracted spectrin was separated from the ghosts pellets by centrifugation at 38 000 rpm for 1 h. Final purification of the crude spectrin was accomplished by chromatography on a Sepharose 2B column (90 × 2.5 cm) equilibrated in 5 mM sodium posphate, 1 mM EDTA, 20 mM KCl, 0.2 mM DTT, and 2 mM NaN_3, pH 7.7. Concentration of spectrin was determined by assuming $A_{280}^{1\%} = 10.1$ (Clarke, 1971).

Band 4.1 was isolated and purified from red cell membranes by the method of Tyler et al. (1979, 1980). The salt-extracted crude, containing band 4.1, band 2.1, band 4.9, and a residual spectrin, was dialyzed against 7.5 mM sodium phosphate, 1 mM EDTA, 20 mM KCl, 0.2 mM DTT, and 2 mM NaN_3, pH 8.0, and loaded onto a DEAE-cellulose (10 × 2.5 cm) column equilibrated in the same buffer. The extract was eluted with the stepped series of salt concentration (Tyler et al., 1980). After the column was loaded, 50, 100, and 200 mM KCl was used to elute minor contaminants, band 4.1, and band 2.1, respectively. Fractions containing purified, concentrated band 4.1 were pooled and dialyzed. Concentration of band 4.1 was determined by using $A_{280}^{1\%} = 8.0$ (Tyler et al., 1980).

Fluorescence Labeling. Eosin-labeled spectrin was prepared by reaction with eosinyl-5-maleimide in 20 mM histidine–HCl buffer, pH 7.4, for 2 h at room temperature (Skou & Esmann, 1980). The reacted spectrin was separated from the free dye by exhausted dialysis against phosphate-buffered saline (5 mM sodium phosphate, pH 7.3, 0.15 M NaCl) at 5 °C. The molar ratio of dye to protein was 2:1.

Band 4.1 was labeled with fluoresceinyl isothocyanate in 10 mM carbonate buffer containing 25 mM NaCl, pH 9.0, for 3 h at room temperature according to the procedure of Nakajima et al. (1979). The removal of the free dye was accomplished by exhaustive dialysis against 5 mM sodium phosphate, pH 7.3, and 0.15 M NaCl at 5 °C. The molar ratio of dye to protein was 1:9. No detectable amount of free dye was observed in both dialyzates.

Fluorescence Measurements. Fluorescence intensities and spectra were measured with a Perkin-Elmer 650-10S spectrofluorometer. Some pilot experiments were performed on an SLM Instruments fluorescence spectrophotometer. Both instruments were equipped with thermostated cell compartments. Preliminary experiments indicated that neither inner filter effects nor the heating effects play any significant role in the chosen concentration range. The excitation wavelength was fixed at 480 nm. Unless otherwise specified, the emission was measured at 520 nm. The band-pass of both the excitation and the emission monochromators was set for no more than 5 nm. The relative quenching of the energy donor (Q_R) was calculated from the expression:

$$Q_R = \frac{F_D + F_A - F_{DA}}{F_D + F_A} \quad (1)$$

where F_D and F_A are the fluorescence intensities of the donor and acceptor, respectively; F_{DA} is the fluorescence intensity of the donor in the presence of the acceptor (donor and acceptor mixture).

Calorimetry. Heats of band 4.1–spectrin interactions were determined calorimetrically. An LKB Model 2107-111 batch

[1] Abbreviations: FITC, fluoresceinyl isothiocyanate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SP, spectrin dimer.

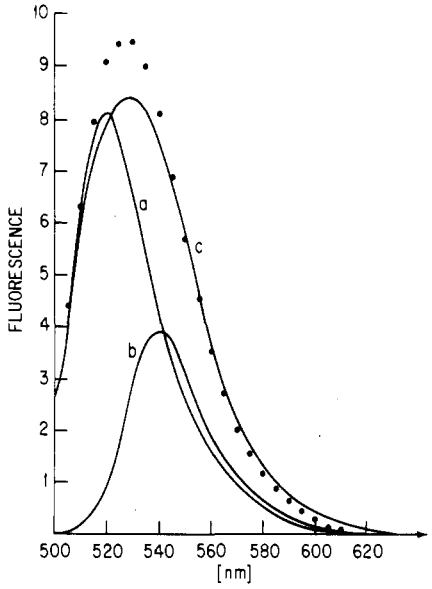

FIGURE 1: Emission spectra of fluorescein–band 4.1 (a), eosin–spectrin dimer (b), and the mixture of fluorescein–band 4.1 and eosin–spectrin (c). Dots represent a numerical sum of spectra a and b. Excitation at 480 nm; 0.15 M NaCl, 5 mM sodium phosphate, pH 7.3; spectrin:band 4.1 ratio 1:1 = 0.3 μM.

microcalorimeter was used in conjunction with the LKB 2107-310 control unit. The calorimetric head was maintained at 30 °C. The gold reaction vessels used with microcalorimeter were of the mixing type. Each compartment of the reference vessel and the reaction vessel was loaded with 1 mL of buffer (5 mM sodium phosphate, pH 7.3, 0.15 M NaCl) and proteins dialyzed against this buffer, respectively.

The electrical calibration curve obtained in the range 0.5–4 mcal was found to be linear. The data were analyzed and interpreted according to generally accepted methodology (Shiao & Sturtevant, 1969; Stoesser & Gill, 1967; Atha & Ackers, 1974). The experimentally measured heats were corrected for heats of reagent dilution and heats of friction obtained by the rotation of the calorimeter unit. The difference of reagent concentration required additional correction for the internal heat of dilution. The obtained amount of enthalpy has been expressed by as kilocalories per initial concentration of the substrate.

Effect of Spectrin–Band 4.1 Complex Formation on pH. A Radiometer PHM82 digital pH meter equiped with glass electrode was used in these studies. Proteins were dialyzed separately against the buffer-free solution containing 0.15 M NaCl. The desired pH 7.25 was obtained by adding aliquotes of diluted NaOH. Efforts were made to remove carbon dioxide from solvents.

RESULTS

Fluorescence resonance energy transfer has been used to directly examine the reaction between spectrin dimer and band 4.1. Fluorescein-labeled band 4.1 and eosin-labeled spectrin dimer were chosen as the energy donor and acceptor, respectively. Figure 1 represents the emission spectra of fluorescein–band 4.1 (a), eosin–spectrin (b), and the mixture of eosin–spectrin and fluorescein–band 4.1 (c), obtained with excitation at 480 nm. Numerical sum of the individual spectra (dots) indicates that spectrum c is not a simple summation of (a) and (b). Significant decrease in the donor fluorescence (donor quenching) and increase of acceptor fluorescence (acceptor enhancement) have been presented in Figure 2, as an effect of wavelength on the relative fluorescence (R_F)

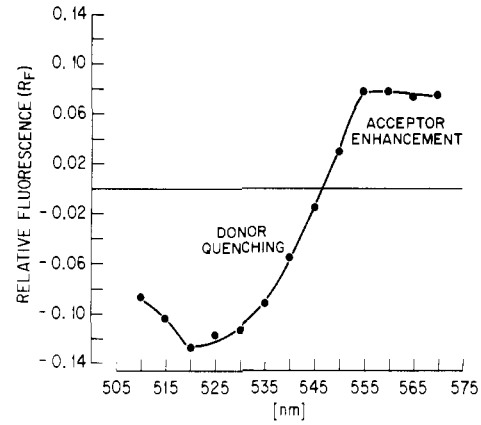

FIGURE 2: Effect of wavelength on the donor quenching and the acceptor enhancement (conditions as in Figure 1).

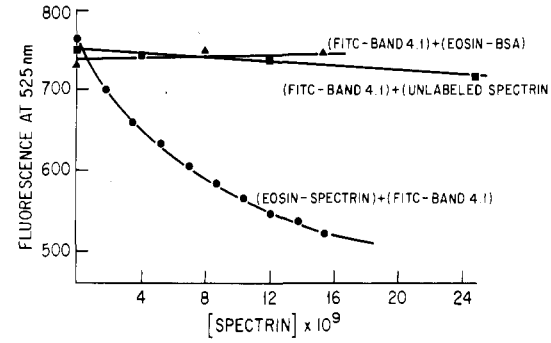

FIGURE 3: Effect of eosin–BSA, unlabeled spectrin, and eosin–spectrin on the fluorescence (arbitrary units) of fluorescein–band 4.1 (FITC–band 4.1), measured at 525 nm (conditions as Figure 1).

calculated from eq 1. Notice that the maximum of the donor quenching occurs at 520 nm. The effect of the local environment on the pK of the amino acid residues in the proximity of the binding site could in part contribute to the observed acceptor enhancement.

In order to eliminate other factors responsible for the observed phenomenon, some control experiments were carried out. Figure 3 summarizes a titration of fluorescein–band 4.1 by eosin–spectrin, eosin–BSA, and unlabeled spectrin measured at 525 nm (excitation 480 nm). Neither eosin–BSA nor unlabeled spectrin were able to quench the donor energy. On the basis of the control experiments, we concluded that changes in the fluorescence observed by the mixture of spectrin and band 4.1 are due to the resonance energy transfer between these two proteins. Presented results have also strongly confirmed the binding of band 4.1 to spectrin dimer and have allowed investigation of the process by measuring changes in the energy transfer. Quantitation of the energy transfer was carried out by measurement of fluorescence intensities at 520 nm (maximum quenching) and calculation of the relative quenching of the energy donor (Q_R) from eq 1.

The effect of band 4.1–fluorescein concentration on the relative quenching by spectrin–eosin at room temperature has been demonstrated in Figure 4. The concentration of spectrin–eosin was kept constant (0.3 μM). The binding isotherm profile (Figure 4) approaches a saturation with increasing total band 4.1–fluorescein concentration at a level corresponding to a mole ratio of 1:1 (band 4.1/spectrin dimer). The stoichiometry of spectrin dimer–band 4.1 interaction obtained directly from fluorescence data has allowed us to establish the equilibrium scheme:

$$[\text{SP}] + [\text{band 4.1}] \underset{K_d}{\overset{K_a}{\rightleftharpoons}} [\text{SP–band 4.1}]$$

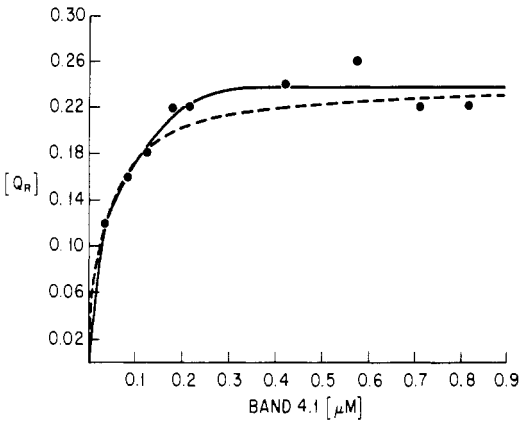

FIGURE 4: Effect of fluorescein–band 4.1 concentration on the relative quenching (Q_R) by eosin–spectrin. The experimental data were fitted with a theoretical value of $K_a = 8.6 \times 10^7$ M^{-1} (see eq 2) (dashed line). [eosin–SP] = 0.3 μM; conditions were as in Figure 1.

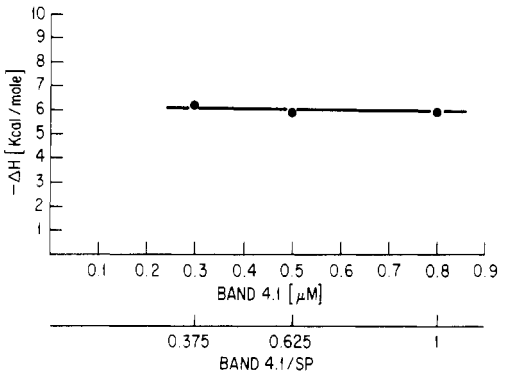

FIGURE 5: Effect of band 4.1 concentration on the enthalpy of reaction with spectrin dimer: [SP] = 0.8 μM; temperature 30 °C; 0.15 M NaCl and 5 mM sodium phosphate, pH 7.30.

Taking into account the above scheme, the association equilibrium constant (K_a) can be expressed (Elbaum & Herskovits, 1974) as

$$K_a = \frac{\alpha}{1-\alpha}\frac{1}{C_T^{4.1}C_t^{SP}} \qquad \alpha = Q_{Rx}/Q_{Rs} \qquad (2)$$

where Q_{Rx} is the relative quenching at a given band 4.1 concentration, Q_{Rs} is the relative quenching at saturation level, and $C_T^{4.1}$ and C_T^{SP} are total concentrations of band 4.1 and spectrin dimer, respectively.

The experimental data were fitted to give the best value for K_a (dashed curve on Figure 4). K_a was found to be 8.6×10^7 M^{-1}, corresponding to the association free energy of −10.8 kcal mol^{-1} ($\Delta F_a = -RT \ln K_a$).

The association constant for the formation of the complex between spectrin dimer and band 4.1 has been previously determined by pelleting in the ultracentrifuge and found to be 5×10^6 M^{-1} (Wolfe et al., 1982) or 1×10^7 M^{-1} (Tyler et al., 1980).

We have determined the contribution of enhalpy to the association free energy by two independent methods: (a) van't Hoff plot and (b) direct microcalorimetric measurement. The effect of temperature (4–30 °C range) on the relative quenching of band 4.1–fluorescein by spectrin–eosin was found, and we attempted to determine the enthalpy of reaction by the van't Hoff method. However, the temperature was found to affect the stability of band 4.1–fluorescein complex, so the data obtained in this manner became unreliable. Thus, we decided to determine the enthalpy of reaction directly from microcalorimetric measurements. The effect of band 4.1

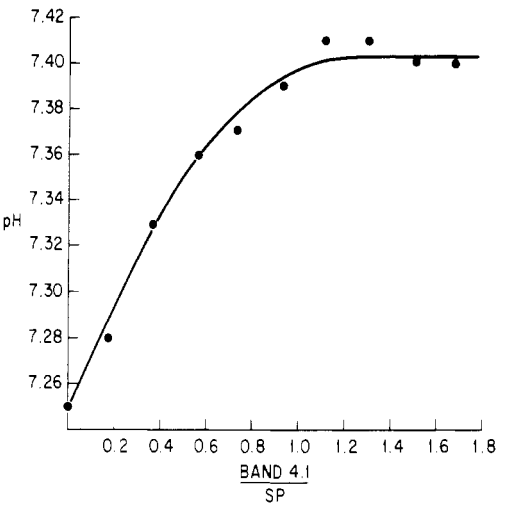

FIGURE 6: Effect of band 4.1 concentration on the pH of spectrin–band 4.1 complex: [SP] = 0.75 μM; 0.15 M NaCl (buffer-free solutions); temperature 25 °C.

concentration on the enthalpy of the reaction between band 4.1 and spectrin dimer has been presented on Figure 5. The concentration of spectrin was kept constant (0.8 μM) in all calorimetric runs. In the experimental range, the enthalpy was independent of the spectrin/band 4.1 mole ratio (Figure 5) and was found to be −6 kcal mol^{-1}. No detectable tetramerization of spectrin has been observed under our experimental conditions as judged by light scattering measurements.

Taking into account the previously mentioned value of the association free energy (−10.8 kcal mol^{-1}), we can calculate the entropy of the reaction. The entropic contribution is responsible for almost 50% of the free energy. This suggests that the protein environment participates in the formation of the spectrin–band 4.1 complex. To examine this possibility, the effect of the complex formation on pH was studied. The spectrin dimer (0.75 μM in buffer free solution) was titrated by band 4.1 in the buffer-free solution, and the pH was measured. Results have been shown in Figure 6. The pH was found to increase with the band 4.1/spectrin ratio (Figure 6), and it strongly suggests that proton uptake is involved in formation of the spectrin dimer–band 4.1 complex. Thus, the considered reaction can be described by following the equilibrium scheme:

$$[SP] + [\text{band } 4.1] \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} [SP\text{–band } 4.1\text{–}H^+]$$

It is interesting to observe that the binding profile (Figure 6) approaches a plateau at a level corresponding to a mole ratio of 1:1 (band 4.1/spectrin). Notice that saturation was reached at the same mole ratio regardless of the presence of the labels on the surface of proteins (Figure 4). This consistence indirectly shows that labeling of spectrin and band 4.1 did not alter the stoichiometry of the reaction.

In order to examine the reversibility of the reaction between band 4.1–fluorescein and spectrin–eosin, unlabeled spectrin was introduced into the system. The effect of the unlabeled spectrin concentration on the relative quenching of band 4.1–fluorescein and the spectrin–eosin mixture (mole ratio 1:1) has been presented in Figure 7. The relative quenching was found to decrease with unlabeled spectrin concentration, and it suggests the replacement of the labeled by unlabeled spectrin.

Discussion

We have determined the thermodynamics of reaction between dimeric spectrin and band 4.1 under the concentration

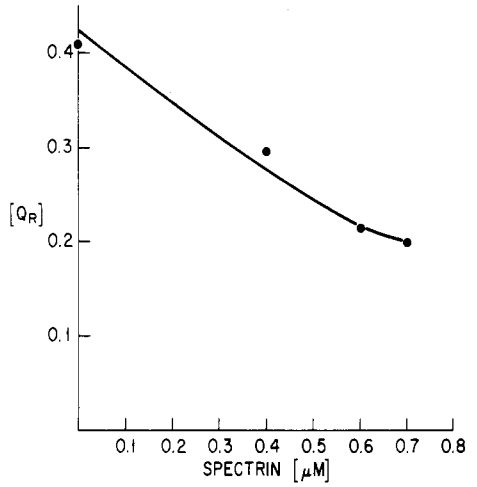

FIGURE 7: Effect of unlabeled spectrin concentration on the relative quenching of fluorescein–band 4.1 and eosin–spectrin complexes (spectrin:band 4.1 ratio = 1:1) (conditions as in Figure 1).

Table I: Effect of NaOH (4 mM) on Rate of Spectrin–Band 4.1 Complex Dissociation[a]

time (min)	Q_R at 520 nm
2	0.41
30	0.23
40	0.11
60	0.10
90	0.0

[a] Spectrin:band 4.1 ratio of 1:1 (0.2 μM); 0.15 M NaCl and 5 mM sodium phosphate, pH 7.3.

ratio range that is pysiologically relevant. The most interesting feature of the binding reaction is the direct contribution of the environment to the free energy of the spectrin–band 4.1 complex formation. The interaction between the proteins results in an uptake of protons (Figure 6). Consistent with this observation is the fact that addition of an aliquot of NaOH dissociates the complex (Table I). Similar conclusions could be obtained by comparing the relative contribution of the enthalpy of the reaction (−6 kcal/mol) and the entropic contribution $-T\Delta S$ (4.8 kcal/mol) to the free energy of binding $\Delta F = -10.8$ kcal/mol. The above reaction has been shown to be reversible (Figure 7). The stochiometry of the reaction between dimeric spectrin and band 4.1 is 1:1 in agreement with others (Tyler et al., 1980).

In order to exclude the possibility that fluorescence labeling of the proteins drastically changes the mechanism of the reaction, we compared the results of the spectrin–band 4.1 binding isotherm of labeled proteins (Figure 4) and the unlabeled material (Figure 6). Both techniques generated indistinguishable stoichiometry of the substrates.

It has been reported by Feo et al. (1980) and Lux & Glader (1981) that individuals homozygous for hereditary elliptocytosis lack band 4.1. This defect has been postulated to be responsible for a significant red cell fragmentation, hemolysis, and osmotic fragility. Alterations within the spectrin molecule have been associated with several cases of heredity elliptocytosis. Liu et al. (1982) have observed an increase concentration of dimeric spectrin in the crude extracts, so a defective dimer–dimer interaction could be responsible for the observed abnormality. An increased heat sensitivity of pyropoikilocytes, as judged by the membrane fragmentation, has been observed by Chang et al. (1979). Abnormality of the spectrin conformation demonstrated by circular dichroism suggests the potential source of the pathology. The above reports support the importance of the spectrin–band 4.1 interaction and the protein conformation in the maintenance of the cytoskeletal integrity.

The interaction between spectrin and band 4.1 is not confined to the red cell membrane. Band 4.1 and spectrin has been reported to be present in other cells (Goodman et al., 1981; Levine & Willanol, 1981; Glenney et al., 1982; Spiegel et al., 1982; Burns et al., 1983). So it is very likely that the information generated by this study will be relevant to other, less understood cellular systems.

We have demonstrated that resonance energy transfer is a valuable technique to study interactions of cytoskeletal proteins. The applicability range of the method is in the range of 30–70 Å, so the majority of the cytoskeletal proteins could be studied by the technique. The combination of resonance energy transfer with the direct determination of the enthalpy of the spectrin–band 4.1 reaction by means of microcalorimetry offers a source of data capable of determining the thermodynamics of the complex formation. Although thermodynamics alone cannot generate a unique model for the complex, it has, however, the ability to exclude all models that will not satisfy the energy requirements. In addition, resonance energy transfer could provide valuable information in understanding the kinetics of cytoskeletal interactions.

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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†

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ABSTRACT: Proteins involved in a structural transition in red blood cell membranes detected at 8 ± 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 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)[1] 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 ± 1.5 °C. Treatments that inhibit cold-induced hypertonic hemolysis and decrease the

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[1] 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(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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