Klionsky, D. J., Brusilow, W. S. A., & Simoni, R. D. (1984) *J. Bacteriol. 160*, 1055–1060.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laget, P. P. (1978) *Arch. Biochem. Biophys. 189*, 122–131.
Laget, P. P., & Smith, J. B. (1979) *Arch. Biochem. Biophys. 197*, 83–89.
Lotscher, H. R., de Jong, C., & Capaldi, R. A. (1984) *Biochemistry 23*, 4140–4143.
Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.
Miki, J., Takeyama, M., Noumi, T., Kanazawa, H., Maeda, M., & Futai, M. (1986) *Arch. Biochem. Biophys. 251*, 458–464.
Neiuwenhuis, F. J. R. M., vander Drift, J. A. M., Vogt, A. B., & vanDam, K. (1974) *Biochim. Biophys. Acta 368*, 461–463.
Nelson, N., Kanner, B. I., & Gutnick, D. L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2720–2724.
Oakley, B. R., Kirsh, D. R., & Morris, N. R. (1980) *Anal. Biochem. 105*, 361–363.
Perlin, D. S., & Senior, A. E. (1985) *Arch. Biochem. Biophys. 236*, 603–611.
Sakamoto, J., & Tonomura, Y. (1983) *J. Biochem. (Tokyo) 93*, 1601–1614.
Satre, M., Bof, M., Issartel, J. P., & Vignais, P. V. (1982) *Biochemistry 21*, 4772–4776.
Schneider, E., & Altendorf, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7279–7283.
Senior, A. E., & Wise, J. G. (1983) *J. Membr. Biol. 73*, 105–124.
Senior, A. E., Downie, J. A., Cox, G. B., Gibson, F., Langman, L., & Fayle (1979) *Biochem. J. 180*, 103–109.
Smith, J. B., & Sternweis, P. C. (1977) *Biochemistry 16*, 306–311.
Smith, J. B., & Sternweis, P. C. (1982) *Arch. Biochem. Biophys. 217*, 376–387.
Soper, J. W., Decker, G. L., & Pederson, P. L. (1979) *J. Biol. Chem. 254*, 11170–11176.
Stan Lotter, H., & Bragg, P. D. (1984) *Arch. Biochem. Biophys. 229*, 320–328.
Sternweis, P. C. (1978) *J. Biol. Chem. 253*, 3123–3128.
Sternweis, P. C., & Smith, J. B. (1977) *Biochemistry 16*, 4020–4025.
Tommasino, M., & Capaldi, R. A. (1985) *Biochemistry 24*, 3972–3976.
Towbin, H., Staehelin, T., & Gorden, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Walker, J. E., & Cozens, A. L. (1986) *Chem. Scr. 26B*, 263–272.
Walker, J. E., Saraste, M., & Gay, N. J. (1984) *Biochim. Biophys. Acta 768*, 164–200.
Wise, J. G., Latchney, R. L., & Senior, A. E. (1981) *J. Biol. Chem. 256*, 10383–10389.
Wise, J. G., Latchney, L. R., Ferguson, A. M., & Senior, A. E. (1984) *Biochemistry 23*, 1426–1432.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.
Yuen, S., Hunkapiller, M. W., Wilson, K. J., & Yuan, P. M. (1986) *User Bull., Appl. Biosyst. 25*, 1–15.

Interactions among Red Cell Membrane Proteins

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ABSTRACT: Interactions between human red cell band 2.1 with spectrin and depleted inside-out vesicles were studied by fluorescence resonance energy transfer and batch microcalorimetry. The band 2.1–spectrin binding isotherm is consistent with a one to one mole ratio. The association constant of 1.4×10^8 M^{-1} corresponds to the association free energy of −11.1 kcal/mol. Under our experimental conditions, the enthalpy of interaction of band 2.1–spectrin was found to be −10.8 kcal/mol and is independent of the protein mole ratio. The calculated entropic factor ($-T\Delta S$ = 0.3 kcal/mol) strongly suggests a predominantly enthalpic character of the reaction. In addition, we investigated the role of band 2.1 on the binding of band 4.1 to spectrin [Podgorski, A., & Elbaum, D. (1985) *Biochemistry 24*, 7871–7876] and concluded that only small, if any, alterations of binding of band 4.1 to spectrin have taken place in the presence or absence of band 2.1. This suggests thermodynamic independence of the binding sites. Although the attachment of the cytoskeletal network to the membrane takes place through, at least, two different interactions, band 2.1–band 3 and 4.1–glycophorin, the relative enthalpy values suggest that band 2.1 contributes significantly more than band 4.1 to the energy of the interaction. In addition, we observed that polymerization of actin is modulated by the cytoskeletons as judged by their effect on the rate of actin polymerization.

The mechanical properties of mammalian erythrocytes are defined by the organized system of proteins located on the cytosolic side of the membrane. Despite the fact that the major cytoskeletal proteins (spectrin, band 4.1, band 2.1, and actin) have been isolated, purified, and, to some extent, characterized, the thermodynamics and the kinetics of the network formation are still an open and unexplored area. Spectrin molecules are the major structural component (approximately 70% by weight) able to bind a single molecule of band 4.1. Two heterodimers of spectrin associated head to head have been postulated to be the predominant form of the protein in the red cell (Ungewickell & Gratzer, 1978). The location of the band 4.1 binding site near the ends of the proteins (Tyler et al., 1979) as judged by electron microscopy renders the tet-

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rameric spectrin bivalent to band 4.1.

While tetrameric spectrin induces an increase in the viscosity of F-actin solutions (Brenner & Korn, 1979), Ungewickell et al. (1979) observed that band 4.1 stimulates F-actin gelation. The data strongly suggest that band 4.1 contributes to the structural stability of the cytoskeleton by enhancing the interaction between spectrin and actin (Elbaum et al., 1984) and by binding directly to band 3 (Pasternack et al., 1985) or other membrane components: cytoplasmic segment of glycophorin (Anderson & Lovrien, 1984) and phosphatidylserine (Sato & Ohnishi, 1983).

Band 2.1 binds to the β subunit of spectrin at a site close to the oligomer binding region. The protein, in turn, binds to the integral membrane (band 3) attaching the cytoskeletal network to the bilayer of phospholipids [see Branton et al. (1981) for a review]. Although some aspects of the thermodynamics of the spectrin–band 4.1 complex formation have been previously studied (Tyler et al., 1980; Ohanian et al., 1984; Cohen & Foley, 1984; Wolfe et al., 1982; Ungewickell et al., 1979; Podgorski & Elbaum, 1985), less is known about the mechanism of spectrin–band 2.1 binding.

In an attempt to determine the magnitude, type, and properties of the interactions responsible for the formation of the cytoskeletal network, we examined several aspects of band 2.1 and band 4.1 binding with the neighboring proteins, hoping that the integration of the available information will contribute to a more complete and holistic energy model of the red cell membrane.

Materials and Methods

Materials. Eosinyl-5-maleimide, fluorescein isothiocyanate I (FITC),[1] and *N*-(1-pyrenyl)iodoacetamide were obtained from Molecular Probes. Actin rabbit muscle, DEAE-cellulose (mesh medium), Folin–Ciocalteu's phenol reagent, bovine serum albumin (BSA), and L-histidine were Sigma products. Dextran T-70 and Sepharose 2B were obtained from Pharmacia. Other common chemicals used for buffers were of at least reagent grade. No more than 1-week-outdated human blood was obtained from the Jacobi Hospital Blood Bank (Bronx, NY).

Protein Preparations. Spectrin dimer was prepared by well-established methods (Ralston, 1976; Tyler et al., 1980): low ionic strength extraction from red cell membranes at 37 °C followed by column chromatography on Sepharose 2B (90 × 2.5 cm) equilibrated in 5 mM sodium phosphate, 1 mM EDTA, 20 mM KCl, 0.2 mM DTT, and 2 mM NaN_3, pH 7.7. The concentration of spectrin, finally dialyzed against 5 mM sodium phosphate and 0.15 M NaCl, pH 7.3, was determined by assuming $A^{1\%}_{280m} = 10.1$ (Clarke, 1971).

Bands 4.1 and 2.1 were isolated and purified from red cell membranes by previously described methods (Tyler et al., 1979, 1980; Podgorski & Elbaum, 1985). The salt-extracted supernatant 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 column (10 × 2.5 cm) equilibrated in the same buffer. The extract was eluted with stepped series of 50, 100, and 200 mM KCl concentration (Tyler et al., 1980; Podgorski & Elbaum, 1985).

Fractions containing purified, concentrated band 4.1 and band 2.1 were pooled separately and finally dialyzed against 5 mM sodium phosphate and 0.15 M NaCl, pH 7.3.

Concentrations of band 4.1 and band 2.1 were determined by using $A1\%_{280nm}$ values of 8.0 and 6.5, respectively (Tyler et al., 1980).

Preparation of Vesicles. The inside-out vesicles (IOVs) and depleted inside-out vesicles (IOVs) were prepared by previously described methods (Bennett & Branton, 1977; Tyler et al., 1979).

IOVs were prepared by incubation of washed erythrocyte ghosts in 40 volumes excess of 0.3 mM sodium phosphate, pH 7.6, at 4 °C for 18 h. Depleted IOVs were obtained by washing erythrocyte ghosts incubated in 30 volumes excess of 0.3 mM sodium phosphate, pH 7.6, at 37 °C for 30 min. This incubation extracted spectrin, actin, and band 4.1, and band 2.1 from the membranes and reduced the ghosts to vesicles smaller than 1 μm (Bennett & Branton, 1977). The membranes were then pelleted by centrifugation at 9000 rpm for 1 h, resuspended in 0.3 mM sodium phosphate, pH 7.6, and passed 3 times through a 27-gauge needle to complete shearing and sealing of vesicles. Sealed and unsealed vesicles were separated over a T-70 dextran barrier (10% w/v in 0.3 mM sodium phosphate and 0.5 mM NaN_3, pH 7.6) by centrifugation at 38 000 rpm for 2 h. The vesicles pelleted at the dextran/buffer interface were washed in 0.7 mM sodium phosphate, 20 mM KCl, and 0.5 mM NaN_3, pH 7.6.

Bands 4.1 and 2.1 were extracted by incubation spectrin depleted inside-out vesicles in 30 volumes excess of 25 mM sodium phosphate, 5 mM EDTA, 1 M KCl, and 0.5 mM NaN_3, pH 8.5, at 37 °C for 1 h (Tyler et al., 1979). After incubation, the vesicles were pelleted and finally washed in 5 mM sodium phosphate and 0.15 M NaCl, pH 7.3. KCl extraction removed more than 65% of band 2.1 and more than 75% of band 4.1 (Tyler et al., 1979).

The protein concentrations in depleted inside-out vesicles were determined by the method of Lowry et al. (1951), using a BSA standard.

Preparation of Labeled Proteins. Bands 4.1 and 2.1 were labeled with fluorescein isothiocyanate as described previously (Nakajima et al., 1979; Podgorski & Elbaum, 1985). The proteins were separately incubated with dye in the presence of 10 mM $NaHCO_3$ and 25 mM NaCl, pH 9.0, for 4 h at room temperature. After reaction, the mixtures were dialyzed against 5 mM sodium phosphate and 0.15 M NaCl, pH 7.3, in a cold room until no detectable amount of free dye was observed in dialysates. The molar ratio of bound fluorescein to both proteins was 0.1–0.2.

Spectrin dimer was labeled with eosinyl-5-maleimide in 20 mM histidine hydrochloride buffer, pH 7.4, for 2 h at room temperature (Skou & Esmann, 1980; Podgorski & Elbaum, 1985). The removal of free dye was accomplished by exhaustive dialysis against 5 mM sodium phosphate and 0.15 M NaCl, pH 7.3, at 5 °C. The molar ratio of band dye to protein was 2.

G-Actin was labeled with *N*-(1-pyrenyl)iodoacetamide as described by Konyama and Mihashi (1981). After removal of unreacted dye, the labeled F-actin was sedimented by centrifugation and resuspended in an aqueous solution containing 2 mM Tris (pH 8.0), 0.2 mM $CaCl_2$, 0.02% NaN_3, and 0.2 mM ATP and was dialyzed against the same buffer at 4 °C during 48 h.

Fluorescence Measurements. A Perkin-Elmer 650-10S spectrofluorometer, equipped with thermostated cell compartments, was used to monitor fluorescence intensities and spectra. Control experiments have shown that neither inner filter effects nor the heating effects play any significant role

[1] Abbreviations: FITC, fluorescein isothiocyanate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; SP, spectrin dimer; IOVs, inverted (inside-out) vesicles; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)-aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

in the chosen concentration range. The excitation monochromator was fixed at 480 nm. Unless otherwise specified, the emission was measured at 520 nm. The band-pass of both excitation and emission monochromators was set for no more than 5 nm.

Fluorescence resonance energy transfer was used to examine the reaction between isolated proteins, as described previously (Podgorski & Elbaum, 1985). Fluorescein-labeled bands 2.1 or 4.1 and eosin-labled spectrin dimer were chosen as the energy donors and acceptor, respectively. Quantitation of the energy transfer was carried out by measurement of fluorescence intensities at maximum quenching (520 nm) and calculation of the relative quenching of the energy donor (Q_R) 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, and F_{DA} is the fluorescence intensity of the donor in the presence of the acceptor.

The binding of isolated proteins to depleted inside-out vesicles (IOVs) was determined by using the fluorescence uptake assay. Eosin-labeled spectrin or fluorescein-labeled band 2.1 was mixed with IOVs and incubated for 1 h at room temperature. Then the mixture was laid over a T-70 dextran barrier (10% w/v) and centrifuged for 2 h at 38 000 rpm. The fluorescence intensities of the supernatant were measured at 520 nm (fluorescein labeling) or 545 nm (eosin labeling). The relative fluorescence was calculated by normalizing results to a full-scale signal.

G-Actin polymerization was monitored by using the same instrument. The excitation and emission wavelengths were 368 and 408 nm, respectively. Polymerization was initiated by adding a small aliquot of polymerization buffer (250 mM KCl, 100 mM PIPES, pH 7.4, 10 mM EGTA, 0.5 mM $CaCl_2$, and 5 mM ATP).

Calorimetric Measurements. An LKB Model 2107-111 batch microcalorimeter in conjunction with the LKB 2107-310 control unit was used in these studies. The working temperature of the calorimetric head was fixed at 30 °C. The two gold reaction vessels consisted of a chamber with an interior dividing wall which extends to two-thirds of the height of the chamber. Each compartment of the reference vessel and the sample vessel was loaded with 1 mL of buffer (5 mM sodium phosphate and 0.15 M NaCl, pH 7.3), against which reactants were dialyzed. The reaction was initiated by rotating the entire instrument, resulting in the mixing of the reactants. The area under the curve of the thermal flow rate as a function of the time has represented the total amount of heat. The data were analyzed and interpreted according to generally accepted methods (Atha & Ackers, 1974; Shiao & Sturtevant, 1969; Stoesser & Gill, 1967).

The corrected heats of interactions (Q_c) were calculated from the expression:

$$Q_c = (Q_R - Q_{FR}) - [(Q_{D1} - Q_{FD1}) + (Q_{D2} - Q_{FD2})] \quad (2)$$

where Q_R is the heat of reaction, Q_{D1} and Q_{D2} are the heats of reagent dilutions, and Q_{FR}, Q_{FD1}, and Q_{FD2} are the heats of friction, measured by a separate rotation of the calorimeter unit after the reaction or dilution runs.

The quantitative performance of the instrument was tested electrically. Calibration showed a linear response in the range 0.5–15 mcal. The uncertainty in the values of heat evolved upon mixing, which arises primarily from slight irregularities in the base line, can be set about 0.1 mcal. This usually amounted to only about 5% of the net evolved heat or less.

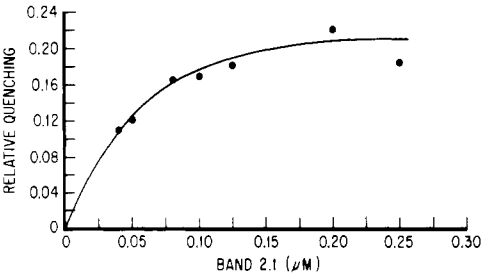

FIGURE 1: Effect of band 2.1–FITC concentration on the relative quenching by spectrin–eosin. Excitation wavelength is 480 nm; emission wavelength is 520 nm; [SP] = 0.2 μM; 0.15 M NaCl/5 mM sodium phosphate, pH 7.3.

The heats of band 2.1–spectrin interactions have been expressed as kilocalories per initial concentration of the substrate. The values representing the heats of reaction between isolated proteins and inside-out vesicles have been expressed as millicalories per milligram of protein in IOVs.

RESULTS

Interaction of Spectrin and Band 2.1. The interactions between spectrin dimer and band 2.1 have been studied by two independent methods: fluorescence resonance energy transfer and microcalorimetry. The effect of band 2.1–fluorescein concentration on the relative quenching (Q_R) by spectrin–eosin at room temperature has been presented on Figure 1. The concentration of spectrin–eosin was kept constant (0.2 μM). The binding isotherm curve shown in Figure 1 approaches a plateau at a level corresponding to a mole ratio of 1:1 (band 2.1/spectrin dimer). This value, obtained directly from the fluorescence data, is consistent with that previously determined by pelleting in the ultracentrifuge (Bennett & Stenbuck, 1979; Tyler et al., 1980). The established stoichiometry of band 2.1–spectrin interaction has allowed us to determine the association equilibrium constant (K_{assoc}), calculated from the expression (Elbaum & Herskovits, 1974; Podgorski & Elbaum, 1985):

$$K_{assoc} = \frac{\alpha}{1 - \alpha} \frac{1}{c_T^{2.1} c_T^{SP}} \qquad \alpha = Q_{Rx}/Q_{Rs} \quad (3)$$

where Q_{Rs} is the relative quenching at saturation level, Q_{Rx} is the relative quenching at a given band 2.1 concentration, and c_T^{SP} and $c_T^{2.1}$ are total concentrations of spectrin dimer and band 2.1, respectively.

K_{assoc} was found to be 1.4×10^8 M^{-1} at the midpoint of the binding isotherm profile (Figure 1), where $Q_{Rx}/Q_{Rs} = \alpha/2$. This value has corresponded to the association free energy of −11.1 kcal mol^{-1} ($\Delta F_{assoc} = -RT \ln K_{assoc}$).

The dissociation equilibrium constant of 10^{-7} M at pH 7.6 determined by pelleting (Tyler et al., 1980) also revealed high affinity of the association between band 2.1 and spectrin dimer.

We have determined the contribution of enthalpy to the association free energy by direct microcalorimetric measurements. The effect of band 2.1 concentration on the enthalpy of the spectrin dimer–band 2.1 interaction has been determined. The concentration of spectrin was kept constant (0.43 μM) in all calorimetric runs. No detectable tetramerization of spectrin was observed under our experimental conditions as evaluated by light-scattering measurements. In the experimental range, the enthalpy was independent of the band 2.1:spectrin mole ratio and was found to be −10.8 kcal mol^{-1} (data not shown).

Taking into account the previously mentioned values of the association free energy (−11.1 kcal mol^{-1}) and the calculated entropic factor ($T\Delta S$ = 0.3 kcal mol^{-1}), our results strongly

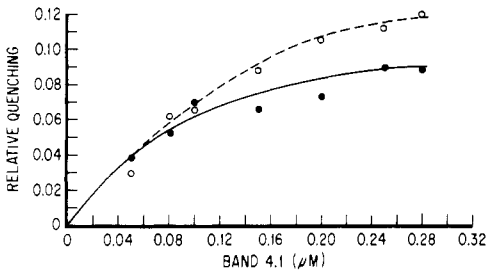

FIGURE 2: Effect of band 4.1–FITC concentration on the relative quenching by spectrin–eosin alone (●) and (spectrin–eosin)–unlabeled band 2.1 complex (○). [SP] = 0.2 μM; spectrin:band 2 ratio of 1:1 = 0.2 μM (conditions as in Figure 1).

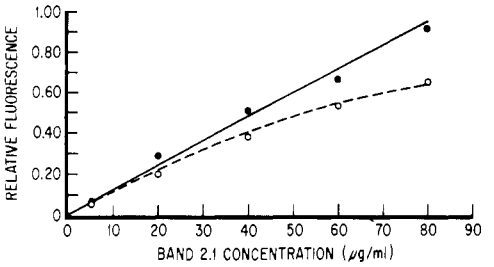

FIGURE 3: Effect of band 2.1–FITC concentration on the interaction with inside-out vesicles (○) and (●) control. [IOVs] = 0.2 mg/mL; 0.15 M NaCl/5 mM sodium phosphate, pH 7.3.

suggest predominantly enthalpic character of the reaction between band 2.1 and spectrin dimer.

We investigated the role of band 2.1 in the binding of band 4.1 to spectrin. The effect of band 4.1–fluorescein concentration on the relative quenching (Q_R) by spectrin–eosin alone and (spectrin–eosin)–unlabeled band 2.1 complex has been presented in Figure 2. The concentration of spectrin and spectrin–band 2.1 complex was kept constant (0.2 μM). The molar ratio of spectrin to band 2.1 was 1:1. Under assumption that binding isotherms in the presence and absence of band 2.1 saturate at the relative quenching levels of 0.15 and 0.09, the association constants, obtained from the midpoints, were found to be 11.5×10^7 and 5.5×10^7 M^{-1}, respectively. These values have corresponded to the association free energies of −11.0 and −10.6 kcal mol^{-1}, respectively. The presented results allowed us to conclude that only small, if any, alterations of binding of band 4.1 to spectrin have taken place in the presence or absence of band 2.1.

Interactions of Isolated Proteins with Depleted Inverted Vesicles. The interactions between pure cytoskeletal proteins and depleted IOVs have been investigated by two independent methods: fluorescence uptake assay and microcalorimetry.

The effect of the band 2.1–fluorescein concentration on the interaction with IOVs has been demonstrated in Figure 3. The association between band 2.1 and IOVs was assayed by measurements of the relative fluorescence (R_F) of the unbounded band 2.1–fluorescence complex (at 520 nm).

The concentration of depleted IOVs was kept constant (0.2 mg/mL). The binding of band 2.1 to KCl-extracted IOVs (from which bands 4.1 and 2.1 had been stripped) was found to increase with increasing total band 2.1–fluorescein concentration (Figure 3). Although this result was expected, it has demonstrated that the relatively simple fluorescein uptake assay is a valuable technique to study interactions between isolated proteins and inside-out vesicles. Therefore, we examined the association between normal band 2.1 and depleted IOVs prepared from a patient (B.U.) whose red cells have been spherocytic. The effects of band 2.1–fluorescein concentration on the interaction with IOVs prepared from spherocytes were found to be experimentally indistinguishable (data not shown).

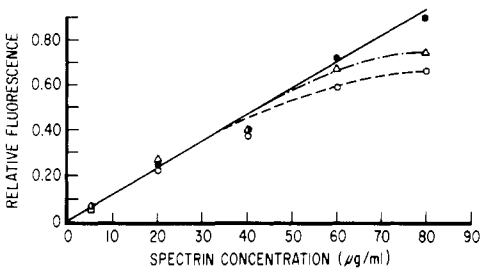

FIGURE 4: Effect of spectrin–eosin concentration on the interactions with IOVs (△), IOVs–band 2.1 complex (○), and (●) control. [IOVs] = 0.15 mg/mL; [band 2.1] = 61 μg/mL; excitation wavelength is 480 nm; emission wavelength is 545 nm; 0.15 M NaCl/5 mM sodium phosphate, pH 7.3.

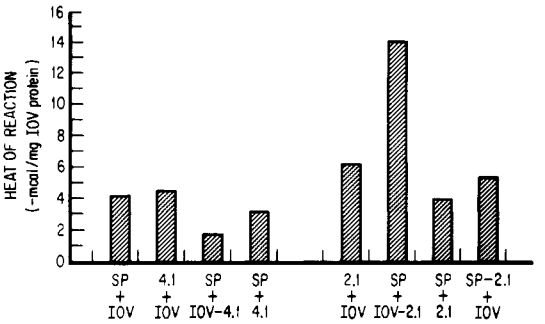

FIGURE 5: Heats of binding of spectrin, band 4.1, and band 2.1 to inside-out vesicles. [IOVs] = 1 mg/mL; [spectrin] = [band 4.1] = [band 2.1] = 0.43 μM; 0.15 M NaCl/5 mM sodium phosphate, pH 7.3.

The preparation of pathological-depleted IOVs and the experimental conditions were the same as for the previously described normal IOVs–band 2.1 system.

The role of band 2.1 in binding spectrin to the depleted IOVs has been examined by the fluorescence uptake assay. The effect of spectrin–eosin concentration on the interactions with IOVs and IOVs–band 2.1 complex has been demonstrated in Figure 4. The concentrations of IOVs and band 2.1 were kept constant (0.15 mg/mL and 61 μg/mL, respectively). The fluorescence intensities of unbounded spectrin–eosin complex were measured at 545 nm. We also found that pure protein 2.1 has stimulated spectrin binding to IOVs (Figure 4). This finding is consistent with the results obtained by using a different technique (Tyler et al., 1979).

The binding of pure proteins to depleted inside-out vesicles has been investigated by direct microcalorimetric measurements. Figure 5 summarizes several experiments designed to determine the heats of interactions between spectrin, band 2.1, band 4.1, and IOVs at various combinations of components. The concentrations of IOVs and isolated proteins were kept constant (1 mg/mL and 0.43 μM, respectively) in all calorimetric runs. The binding of band 2.1 to IOVs was more energetic than that of band 4.1 or spectrin (Figure 5). The heat of association between spectrin and IOVs increased in the presence of band 2.1. When IOVs were preincubated with protein 2.1, the heat of the spectrin–IOV interaction reached an even higher level (Figure 5). Conversely, the preincubation of IOVs with band 4.1 has significantly diminished the heat of spectrin–IOVs interaction (Figure 5). We have assumed that the heat of interaction can be considered as an indicator of the binding energy. Taking this into account, our results strongly suggest that band 2.1 has stimulated the binding of spectrin to the native membrane whereas band 4.1 inhibited the binding.

We previously reported (Podgorski & Elbaum, 1985) that the protein environment has participated in the formation of

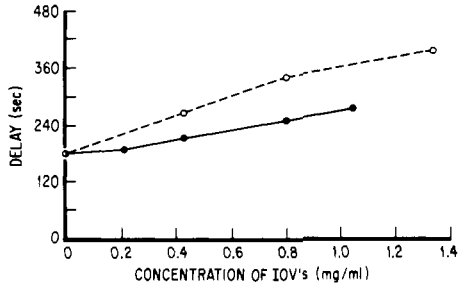

FIGURE 6: Effect of varying concentrations of IOVs (O) and depleted IOVs (●) on the delay of polymerization of G-pyrenylactin (O) (no IOVs added). [G-Pyrenylactin] = 0.9 μM, pH 7.3, 21 °C. To initiate polymerization (t = 0), polymerization buffer was added to make the final buffer condition 250 mM KCl, 100 mM PIPES, pH 7.4, 10 mM EGTA, 0.5 mM $CaCl_2$, and 5 mM ATP.

the spectrin–band 4.1 complex. Thus, we decided to examine the role of environment in the band 2.1–IOV and band 4.1–IOV interactions. The effect of pH on the heats of binding of proteins 2.1 and 4.1 to IOVs has been determined. The concentrations of IOVs (1 mg/mL), band 4.1 (0.43 μM), and band 2.1 (0.43 μM) were kept constant in all calorimetric measurements. The heats of band 2.1–IOV and band 4.1–IOV interactions were found to change in the opposite directions with an increase of pH (data not shown). This may reflect different roles of proteins 2.1 and 4.1 in the organization of the erythrocyte membrane, although the mechanism of the cytoskeletal protein–native membrane–environment interactions remains to be explored.

The effect of IOVs and depleted IOVs on the delay of pyrenylactin polymerization was studied by following the fluorescence changes (Figure 6). Pyrenylactin has been shown by a number of investigators (Tobacman & Korn, 1983) to be a very sensitive probe of actin polymerization. The label reacts specifically with sulfhydryl groups on the surface of the actin molecule and reports local conformational changes induced by formation of actin filaments (Tobacman & Korn, 1983). The presence of IOVs was found to prolong the delay of actin polymerization. Interestingly, depleted IOVs were observed to alter the kinetics of the reaction less significantly then IOVs. In addition, the above effect was found to be pH independent from pH 5.8 to pH 7.8 (data not shown).

Discussion

Band 2.1 is one of the major components of the erythrocyte membrane. Its major function is to link spectrin to the cytoplasmic domain of band 3. It implies that the protein contains at least two binding sites: (a) the spectrin and (b) the band 3 affinity areas.

One of the most important observations which can be made on the basis of the calorimetric results (Figure 5) is that the band 2.1 binding sites are neither independent nor enthalpically comparable in their magnitude. In addition, our observations suggest that the thermodynamics of band 2.1 in the proximity of IOV are distinctively different than in the isolated and purified form. The results imply that caution should be exercised in interpretation of the results obtained from the reconstituted membranes.

Although the attachment of the cytoskeleton to the membrane takes place through at least two different interactions, band 2.1–band 3 and band 4.1–glycophorin (Anderson & Marchesi, 1983), the relative enthalpy values suggest that band 2.1 contributes significantly more than band 4.1 to the energy of the interaction (see Figure 5). It is possible that distinctively different thermodynamics of spectrin–band 4.1 (Podgorski & Elbaum, 1985) and spectrin–band 2.1 could be responsible for this observation. While the entropic contribution to the free energy of the spectrin–band 4.1 interaction accounts for approximately 50% of the binding energy, spectrin–band 2.1 is predominantly enthalpic (out of −11.1 kcal/mol of the free energy, −10.8 kcal/mol is due to the enthalpic contribution).

Interestingly, our findings suggest that interactions between band 4.1 redecorated inside-out vesicles and spectrin are the weakest link of the spectrin attachment to the depleted membranes. However, in light of a substantial $T\Delta S$ contribution to the spectrin–band 4.1 binding, as measured when pure band 4.1 and spectrin were reacted, it is possible that the entropy could contribute to the spectrin–band 4.1 redecorated IOV interactions.

Assuming that the predominant binding site of the band 2.1–spectrin complex with IOVs is band 3 (Weaver et al., 1984) and band 4.1–spectrin binds mostly to major sialoglycoproteins, it is apparent from our results that band 2.1 is responsible for most of the energy of the attachment. It is tempting to speculate that a perturbation or spectrin abnormality in the domain of the band 2.1 binding site with spectrin could significantly destabilize the interaction of the cytoskeletons with the rest of the red cell membrane. In an attempt to examine the possibility that spherocytes could have a defective band 2.1 binding site, we compared the effect of band 2.1 concentration on the interaction with IOVs prepared from normal and pathological red cells. No experimentally significant differences were observed, suggesting either lack of the abnormalities confined to the domain of the binding site or thermodynamic insignificance of the alteration. It is important to emphasize, however, that the above speculations could be complicated by the observation that band 3 could interact with band 4.1 (Pasternak et al., 1985).

We previously observed (Elbaum et al., 1984) that the complex of dimeric spectrin and band 4.1 is capable of regulating the rate of actin polymerization. The presence of this complex shortens the delay of the reaction. However, IOVs and depleted IOVs prolong the rate. A possible basis for the observed difference between the effect of IOVs and the complex on the actin aggregation could be the presence of several other cytoskeletal components: band 2.1, band 3, band 4.9, etc. The above conclusion is supported by the fact that depleted IOVs have effected the actin aggregation less significantly than the vesicles with most of the membrane component intact.

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References

Anderson, R. A., & Lovrien, R. E. (1980) *Nature (London) 307*, 655–658.

Anderson, R. A., & Marchesi, V. T. (1983) *J. Cell Biol. 97*, 297 (Abstr.).

Atha, D. H., & Ackers, G. K. (1974) *Arch. Biochem. Biophys. 164*, 392–407.

Bennett, V., & Branton, D. (1977) *J. Biol. Chem. 252*, 2753–2763.

Bennett, V., & Stenbuck, P. J. (1979) *Nature (London) 280*, 468–473.

Branton, D., Cohen, C. M., & Tyler, J. (1981) *Cell (Cambridge, Mass.) 24*, 24–32.

Brenner, S. L., & Korn, E. D. (1979) *J. Biol. Chem. 254*, 8620–8627.

Clarke, M. (1971) *Biochem. Biophys. Res. Commun. 45*, 1063-1070.
Cohen, C. M., & Foley, S. F. (1984) *Biochemistry 23*, 6091-6098.
Elbaum, D., & Herskovits, T. T. (1974) *Biochemistry 13*, 1268-1278.
Elbaum, D., Mimms, L. T., & Branton, D. (1984) *Biochemistry 23*, 4813-4816.
Kouyma, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33-38.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Nakajima, M., Yoshimoto, R., Irimura, T., & Osawa, T. (1979) *J. Biochem. (Tokyo) 86*, 583-586.
Ohanian, V., Wolfe, C. L., John, K. M., Pinder, J. C., Lux, S. E., & Gratzer, W. B. (1984) *Biochemistry 23*, 4416-4420.
Pasternack, G. R., Anderson, R. A., Leto, T. L., & Marchesi, V. T. (1985) *J. Biol. Chem. 260*, 3676-3683.
Podgorski, A., & Elbaum, D. (1985) *Biochemistry 24*, 7871-7876.
Ralston, G. B. (1976) *Biochim. Biophys. Acta 443*, 387-393.
Sato, S. B., & Ohnishi, S. (1983) *Eur. J. Biochem. 130*, 19-26.
Shiao, D. D. F., & Sturtevant, J. M. (1969) *Biochemistry 8*, 4910-4917.
Skou, J. C., & Esmann, M. (1980) *Biochim. Biophys. Acta 601*, 386-402.
Stoesser, P. R., & Gill, S. J. (1967) *J. Phys. Chem. 71*, 564-567.
Tobacman, L. S., & Korn, E. D. (1983) *J. Biol. Chem. 258*, 3207-3214.
Tyler, J. M., Hargreaves, W. R., & Branton, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5192-5196.
Tyler, J., Reinhardt, B. N., & Branton, D. (1980) *J. Biol. Chem. 255*, 7034-7039.
Ungewickell, E., & Gratzer, W. (1978) *Eur. J. Biochem. 88*, 379-385.
Ungewickell, E., Bennett, P. M., Calvert, R., Ohanian, V., & Gratzer, W. B. (1979) *Nature (London) 280*, 811-814.
Weaver, D. C., Pasternack, G. R., & Marchesi, V. T. (1984) *J. Biol. Chem. 259*, 6170-6175.
Wolfe, L. C., John, K. M., Falcone, J. C., Byrne, A. M., & Lux, S. E. (1982) *N. Engl. J. Med. 307*, 1367-1374.

Identification of the Protein 4.1 Binding Site to Phosphatidylserine Vesicles†

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ABSTRACT: Previous studies have shown that protein 4.1 is a multifunctional protein that binds to spectrin, actin, glycophorins, the anion channel protein, and phosphatidylserine (PS). In this report, we have characterized the binding of protein 4.1 and its major proteolytic fragments to phospholipid vesicles. Pure ^{125}I-labeled protein 4.1 was incubated with PS liposomes, and the free protein 4.1 was separated by ultracentrifugation. Protein 4.1 bound to PS liposomes with a high affinity. At saturation, there was 9×10^{-3} pmol of protein 4.1 bound/pmol of PS with a K_d of 3.3×10^{-7} M. When the protein 4.1 containing liposomes were examined in an electron microscope, the protein 4.1 was found uniformly decorating the vesicles in a rosettelike fashion. Among peripheral membrane proteins tested (spectrin, actin, ankyrin, and protein 4.1), protein 4.1 showed the highest level of binding to PS. The binding of protein 4.1 to PS, one of the principal phospholipids of the inner half of the lipid bilayer, was considerably higher than the binding to phosphatidylcholine, that is principally located in the outer half of the lipid bilayer. To identify the structural domain of protein 4.1 involved in binding to the phospholipids, a mixture of proteolytic fragments of protein 4.1 was incubated with PS liposomes. The liposomes selectively retained the 30-kilodalton (kDa) basic domain of the protein, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/isoelectric focusing. The 30-kDa fragment was purified from chymotryptic digests of protein 4.1 by ion-exchange chromatography on DEAE 52. The purified 30-kDa peptide of protein 4.1 competitively inhibited the binding of ^{125}I-labeled protein 4.1 to PS. These data indicate that protein 4.1 is capable of forming high-affinity associations with PS. These associations may be important in the maintenance of normal red cell structure and function.

The red blood cell membrane skeleton contains four major proteins: spectrin, actin, protein 4.1, and protein 4.9 (Cohen, 1983). Protein 4.1 is composed of two very similar polypeptide chains of 80 and 78 kilodaltons (kDa) which have essentially identical peptide maps (Goodman et al., 1982). The larger polypeptide differs from the smaller by having an extension at its carboxyl terminus (Leto & Marchesi, 1984). The structure of protein 4.1 was recently studied by limited proteolysis employing chymotrypsin (Leto & Marchesi, 1984). Protein 4.1 was cleaved primarily in three central locations which generated intermediate-size peptides that were found to represent specific structural and functional domains of the original molecule. The protein 4.1 molecule displays unusual polarity, and its 56- and 46-kDa chymotryptic digests contain the 30-kDa basic peptide that appears to be resistant to further digestion by chymotrypsin (Leto & Marchesi, 1984). Protein

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