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ESR studies of the erythrocyte membrane skeletal protein network: influence of the state of aggregation of spectrin on the physical state of membrane proteins, bilayer lipids, and cell surface carbohydrates

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The stability of the human erythrocyte membrane skeletal network is reported to be dependent on the state of aggregation of spectrin and decreased or increased by polyphosphate anions or the polyamine, spermine, respectively. We have employed polyacrylamide gel electrophoresis and electron spin resonance (ESR) utilizing spin labels specific for membrane proteins, bilayer lipids, or cell-surface sialic acid in order to gain insight into these observations and into the reliability of the ESR spectra of the protein-specific spin label used to correctly report the interactions of the skeletal protein network. The major findings are: (1) We confirm previous reports that the preferred state of spectrin aggregation in the skeletal network is tetrameric and that spectrin can be reversibly transformed to dimeric spectrin and back to tetrameric spectrin on the membrane. (2) The ESR spectra of the protein specific maleimide spin label employed accurately reflect the state of aggregation of spectrin. (3) As dimeric spectrin is increased on the membrane or when 2,3-bisphosphoglycerate was added to spin-labeled membranes, increased segmental motion of protein spin label binding sites reflecting decreased protein-protein interactions in the skeletal network is observed ($P < 0.002$ and $P < 0.005$, respectively). (4) Conversely, as protein-protein interactions between skeletal proteins or between skeletal proteins and the bilayer are increased by spermine (reflected in the total inability to extract spectrin from the membrane in contrast to control membranes), highly decreased segmental motion of the protein specific spin label binding sites is observed ($P < 0.005$). (5) The dimeric-tetrameric state of spectrin aggregation on the membrane does not have influence on the order or motion of bilayer lipids nor on the rotational rate of spin-labeled, cell-surface sialic acid, a result also observed when protein-protein interactions were decreased by 2,3-bisphosphoglycerate. In contrast, increased protein-protein interactions by addition of spermine produced a small, but significant, increase in order and decrease in motion of bilayer lipids near the membrane surface as well as a nearly 40% decrease in the apparent rotational correlation time of spin labeled, cell surface sialic acid ($P < 0.002$). These latter observations are discussed with reference to possible associations of phospholipids and the major, transmembrane sialoglycoprotein with the skeletal protein network.

Introduction

The cytoplasmic side of the erythrocyte membrane is covered by a meshwork of proteins known

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as the skeletal network A number of membrane roles have been found for the skeletal network including cell shape, deformability, substrates for certain membrane enzymes (e g , protein kinase), and control of the lateral diffusion of transmembrane glycoproteins [1–5] The major skeletal protein is spectrin which consists of intertwined α- and β-chains to form heterodimers that associate to form head-to-head tetramers [6] Higher oligomers of spectrin may exist [7] The skeleton consisting of spectrin, actin, band 4 1, and possibly band 4 9 [1] is attached to the bilayer by a linkage of spectrin to a fraction of the major transmembrane protein band 3 through band 2 1 [8,9] Band 4 1 is apparently attached to the bilayer thereby providing a second skeletal binding site to the membrane although the exact nature of the linkage is not fully understood Whether the linkage is through the minor glycoprotein (PAS-2) [10], the major sialoglycoprotein (glycophorin A) [11], both of these glycoproteins, or negatively charged phospholipids [12,13] is not known for certain

Genetic hemolytic anemias with increased amounts of dimeric spectrin or altered spectrin-band 4 1 interactions, both with consequent skeletal protein instability are known [14–16] The polyphosphate metabolite, 2,3-bisphosphoglycerate, is reported to disrupt skeletal protein associations and permit a faster lateral translational diffusion of transmembrane glycoproteins in the plane of the bilayer [5,17]

The electron spin resonance technique of spin labeling employing spin labels specific for the lipid bilayer [18], cell-surface carbohydrates [19–22], and membrane proteins [23–26] has provided insight into membrane structure and function in normal [27,28] and disease state [23,29,30] membranes An often used protein-specific spin label, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) is covalently bound primarily to skeletal proteins (spectrin, actin, Band 4 1), band 2 1, and the cytoplasmic pole of band 3 [23,24,26,31] In order to gain more insight into the reliability of the ESR spectra of MAL-6 to accurately reflect membrane protein-protein interactions occurring in the membrane skeleton, to determine if the state of aggregation of spectrin has any effect on the physical state of membrane proteins, bilayer lipids, and cell-surface sialic acid, and to investigate the spatial relationship of glycophorin A with the skeletal network, a series of spin-labeling experiments was performed

Methods

Chemicals

Phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 2,3-DPG, spermine, and 2-mercaptoethanol were obtained from Sigma All electrophoresis chemicals were electrophoresis grade from Bio-Rad, United States Biochemical Corporation, or Eastman Chemicals The spin labels, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-NS) were obtained from Aldrich, while the perdeuterated, ^{15}N-spin label, 2,2,6,6-[$^{2}H_{12}$]tetramethyl-4-amino[$^{2}H_{5}$,^{15}N]piperidine-1-oxyl ([^{15}N]Tempamine-d_{17}) was obtained from Merck Stable Isotopes Ultraviolet spectroscopy and melting point analyses showed that no isomaleimide was present in the MAL-6 preparation

Preparation of membranes and spin labeling

Blood was obtained by venipuncture into heparinized tubes, immediately put on ice, and processed within 30 min of drawing blood Intact cells were isolated by centrifugation at 4°C at $600 \times g$ and three subsequent resuspensions and washings in PBS(P) buffer (5 mM sodium phosphate, 150 mM NaCl, pH 8 0 buffer containing 0 1 mM TPCK, 0 1 mM TLCK, and 0 1 mM PMSF) Ghosts were obtained by osmotic lysis of washed cells at 4°C using 5P8(P) buffer (5 mM sodium phosphate buffer, pH 8 0 containing all the protease inhibitors used in PBS (P) at the concentrations noted) and subsequent washings at 4°C and $27\,000 \times g$ in this buffer until hemoglobin-free ghosts were obtained

Selective spin labeling of membrane proteins and sialic acid with MAL-6 and [^{15}N]Tempamine-d_{17}, respectively, were performed at 4°C as previously described [19,23,24] prior to any spectrin transformations Spin labeling of the lipid bilayer with 5-NS was performed as previously described

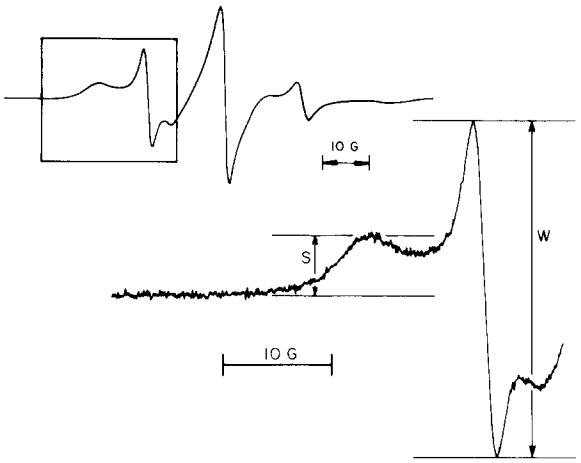

Fig 1 A typical spectrum of the $M_I = +1$ low-field lines of the ESR spectrum of the piperidine maleimide spin label is presented The spectral amplitudes of the spin label covalently bound to strongly and weakly immobilized binding sites are indicated by *S* and *W*, respectively Fresh erythrocyte ghosts were labeled overnight at 4°C by MAL-6 in 5P8 After five washes with 5P8 buffer to remove excess spin label the spectra of the spin-labeled ghosts were recorded with a sweep width of 100 G and modulation amplitude and microwave power incident on the resonant cavity of 0 32 G and 14 mW respectively using a Varian E-109 ESR spectrometer The region of the entire spectrum (inset) corresponding to that illustrated is contained in the box

[23] after spectrin transformations since heating at 37°C required in the latter procedure may have led to distribution of the spin label not normally observed [23]

All ESR spectra were recorded on a Varian E-109 Century Series ESR spectrometer equipped with a TM mode cavity and associated quartz aqueous sample cell Instrumental parameters are noted in the legends to Fig 1

For protein labeled samples, no incubation or warmup was conducted after the dimer-tetramer transformations had been executed The samples were removed from ice and the ESR spectra were immediately recorded The S and W peaks of the $M_I = +1$ nitrogen hyperfine line were recorded first, then the baseline was recorded Each spectrum required approx 7–9 min to acquire from the time of the removal of the sample from the ice A period in which the sample temperature was approx 8°C Transformed ghosts (0 3 ml) were allowed to incubate for 30 min at room temperature with the lipid spin labels before recording the ESR spectra Ghosts whose sialic acid residues were labeled with [^{15}N]Tempamine-d_{17} were allowed to incubate for 15 min at room temperature before recording the ESR spectra

Modification of the state of aggregation of spectrin on the membrane and separately in solution

The state of aggregation of spectrin on the erythrocyte membrane was modulated as described by Liu and Palek [32] Briefly, hemoglobin-free ghosts, prepared as described above were washed two times in PBS(P) or 5,5P8(P) (which is 5P8(P) containing 5 mM NaCl) and were then resuspended at 37°C in PBS(P) or 5,5P8(P) to ten times the original volume of the ghosts The incubation was allowed to proceed for 15–20 min at 37°C The ghosts were then washed three times in ice-cold 5P8 and resuspended in ice-cold 5P8 to a protein content of 3 mg/ml Thus, all ESR spectra were recorded for ghosts in 5P8 buffer PBS(P) treatment has been shown to preserve the in vivo spectrin dimer-tetramer ratio and to restore the dimer-tetramer ratio of ghosts bearing predominantly dimeric spectrin to the original value [32] 5,5P8(P) treatment, however, increases the dimer-tetramer ratio [32] Spectrin on the membranes modified as above and then kept at 4°C is reported to be kinetically trapped in the particular state of aggregation desired [33]

In order to confirm these transformations, non SDS, non-reducing polyacrylamide gel electrophoresis (PAGE) of spectrin extracted from ghosts after PBS(P) or 5 5P8(P) treatments was performed at 4°C using 50 V for 48 h as described in Ref 34 A 3 0% acrylamide gel was employed The gels were stained in a 0 5% Coomassie blue solution and were scanned at 580 nm by an ISCO spectrophotometric gel scanner In order to extract spectrin, the ghosts were washed one time in spectrin-removal buffer (SR buffer 0 1 mM sodium phosphate, 0 1 mM EDTA, 0 1 mM TPCK, 0 1 mM TLCK, and 0 1 mM 2-mercaptoethanol (pH 7 6)) by centrifugation at 27000 × *g* at 4°C The supernatant was then aspirated and the ghosts were resuspended to twice their original volume in ice-cold spectrin-removal buffer The extraction proceeded for approx 40 h at 4°C The residual membrane pellet was then packed by centrifugation for 1 h at 27000 × *g* at 4°C The supernatant

was carefully removed in order to avoid contamination by any protein in the pellet and stored at 4°C for protein and PAGE analysis

The effect of dimer-tetramer transformations on the dynamics of purified spectrin in solution was also monitored Ghosts were spin labeled with MAL-6, washed one time in 0 1 mM sodium phosphate, 0 1 mM EDTA, 0 1 mM TLCK, 0 1 mM TPCK, pH 7 6 buffer and resuspended to the original volume therein The ghosts were incubated for 30 min at 35°C in this buffer and centrifuged at 4°C at $27000 \times g$ for 90 min in order to separate the extracted spectrin from the residual membrane pellet No 2-mercaptoethanol was used in the extraction buffer so as not to reduce the nitroxide spin label The supernatants of the extractions were pooled and concentrated at 4°C by membrane dialysis ($m_{r\ cut\text{-}off} = 10000$) to a volume of approx 2–3 ml This concentrated supernatant was chromatographed at 4°C on a Sephacryl 200 column (40 cm × 2 cm) previously equilibrated with phosphate-buffered saline (pH 7 5) containing 5 mM EDTA 2-ml fractions were collected at a flow rate of 30–40 ml/h Spectrin, predominantly in the dimeric state, came off of the column at the end of the void volume The fractions containing spectrin were pooled and concentrated to 1–2 ml by membrane dialysis Spectrin, concentrated in this fashion, was divided into two aliquots One aliquot was stored at 4°C for 20 min to preserve its dimeric state The other aliquot, still dissolved in the high ionic strength elution buffer, was heated for 20 min at 37°C, thereby transforming dimeric spectrin to tetrameric spectrin Both samples were then put on ice The ESR spectrum of each sample was recorded immediately after it was removed from the ice

2,3-Bisphosphoglycerate was prepared in 5P8 at initial concentrations of 5–20 mM The pH remained at 8 0 A control solution was prepared by adding Na_2HPO_4 to 5P8 to a concentration 5-times that of 2,3-bisphosphoglycerate The pH was adjusted to 8 0 by adding approx 1 μl of 1 M HCl In this manner, the ionic strength of the control solution approximated and the pH equaled that of the 2,3-bisphosphoglycerate solution 60 μl of the appropriate 2,3-bisphosphoglycerate solution was incubated with 240 μl of spin-labeled ghosts for 15 min at room temperature All ESR spectra employing 2,3-bisphosphoglycerate were recorded at room temperature

Spermine solutions were prepared in 5P8 at known concentrations of 3 0–9 0 mM The pH was adjusted to 8 0 by adding a known volume, V_0, of 1 M HCl The error introduced in the concentration of spermine was typically less than 1% A control solution was prepared by adding the volume V_0 of a 0 5 M NaCl solution to a volume of 5P8 equivalent to the volume of the spermine solution prior to HCl addition The resulting concentration of NaCl in the control solution was typically 8–10 mM

Four volumes of ghosts in 5P8 kept at 4°C were allowed to equilibrate at room temperature for 15 min One volume of the spermine solution (final spermine concentration = 0 6–1 8 mM) or of the control solution, previously warmed to room temperature, was added for 30 min prior to spectrin removal or ESR studies

In order to study the effect of spermine on the ease of spectrin removal, this protein was extracted from control or spermine-treated membranes as described above The supernatant was carefully removed in order to avoid contamination by any protein in the pellet and stored at 4°C for PAGE analysis if they were to be performed on the same day Otherwise, all samples were frozen in liquid nitrogen and stored at −10°C until used PAGE was performed according to the method of Laemmli [35] employing an 11 cm resolution gel of 10 0% acrylamide and a stacking gel of 3 5% acrylamide Electrophoresis was carried out at room temperature with 2 5 mA per gel tube

Erythrocyte membrane lipids were extracted from freshly prepared ghosts by the methods of Folch-Pi et al [36]

Results

On the erythrocyte membrane as part of the skeletal network, spectrin exists primarily in the tetrameric state of aggregation [32], although higher oligomers may exist [7] This previous observation and the relative ease by which dimeric and tetrameric spectrin could be interconverted on the membrane by ionic strength and temperature was completely confirmed in the present studies by non-reducing, non-SDS PAGE (data not shown)

In order to determine if the physical state of membrane proteins, lipids, and carbohydrates were affected by the state of spectrin aggregation in the skeletal network, spin labeling studies employing probes specific for each membrane environment were performed

Fig 1 shows a typical ESR spectrum of erythrocyte membrane prtoeins spin labeled with the protein-specific maleimide spin label, MAL-6 Approximately 70–90% of the MAL-6 binding sites are on skeletal proteins and the cytoplasmic pole of the transmembrane protein, band 3 [23,24,26,31] This spectrum is analyzed by the W/S ratio of the spectral amplitudes of the $M_I = +1$ lines as extensively discussed previously [23–25] The W/S ratio has been shown to sensitive to a number of experimental variables [23,24] for which controlled samples were examined in the present study Other workers have indicated that spin labeling erythrocyte ghost membranes with MAL-6 by our procedure [24] resulted in highly reproducible spectra that could be employed with confidence [25] In general, on a membrane or for the same incorporation of MAL-6 in different samples of the same membrane, the higher the W/S ratio, the greater the segmental motion of MAL-6 binding sites on the proteins [23–25]

The state of aggregation of spectrin on erythrocyte membranes previously spin labeled with MAL-6 was varied No differences in the gel profiles of non-reducing, non-SDS PAGE gels of spectrin isolated from spin-labeled membranes compared to that of spectrin isolated from non-spin-labeled membranes were found (data not shown) in agreement with previous publications [26] Table I presents the ESR results No statistically significant difference in the W/S ratio of MAL-6 principally covalently bound to membrane skeletal proteins in erythrocyte ghosts prepared at 4°C from fresh intact cells relative to that in erythrocyte ghosts in which spectrin was forced to the tetrameric state of aggregation was observed This finding is consistent with the gel profiles that we obtained confirming the results of Liu and Palek who suggest that tetrameric spectrin is the preferred state of aggregation on the membrane [32] and further demonstrates that simply heating ghosts at 37°C for 20 min in PBS(P) does not alter the physical state of membrane proteins as judged by the W/S ratio of MAL-6 over that of ghosts prepared from intact cells

TABLE I

EFFECT OF THE STATE OF AGGREGATION OF SPECTRIN ON THE PHYSICAL STATE OF MEMBRANE PROTEINS IN HUMAN ERYTHROCYTE GHOSTS SPIN LABELED BY MAL-6

All transformations are performed as in Ref 32 and described in Methods and confirmed by non-reducing non-SDS PAGE The W/S ratios are presented as means ± S E (N = number of samples)

Transformation	W/S ratio (N)
1 None	4 92 ± 0 19(13)
2 Ghosts → tetrameric spectrin	4 78 ± 0 20(14) [a]
3 Ghosts → increased dimeric spectrin	5 94 ± 0 26(14) [b]
4 Ghost → tetrameric spectrin → dimeric spectrin → tetrameric spectrin	5 02 ± 0 20 (8) [a]

[a] Not significantly different from that in ghosts with no spectrin transformation

[b] $P < 0\,002$ (Student s two tailed t-test) relative to ghosts with tetrameric spectrin

However, upon increasing the proportion of dimeric spectrin on the erythrocyte membrane by methods described above [32], a greatly increased W/S ratio is found ($P < 0\,002$) indicating increased segmental motion of protein spin-label binding sites and an altered physical state of membrane proteins (Table I) This approx 24% increase in the W/S ratio of MAL-6 was completely reversible If spin-labeled ghosts were cycled through the steps by which spectrin was forced to principally the tetrameric form, then the dimeric form increased, and then finally back to nearly all tetrameric form (all confirmed by PAGE), the W/S ratio was statistically indistinguishable from ghosts in which spectrin had only been forced to be primarily in the tetrameric state of aggregation (Table I)

It can be seen that the W/S ratio of MAL-6 reflecting the physical state of spin-labeled proteins paralleled the state of aggregation of spectrin However, we wished to determine if this ESR parameter of MAL-6 was reflecting the alteration in the conformation of spectrin itself or perhaps reflecting the decreased protein-protein interactions in the erythrocyte protein skeletal network known to occur upon increased amounts of di-

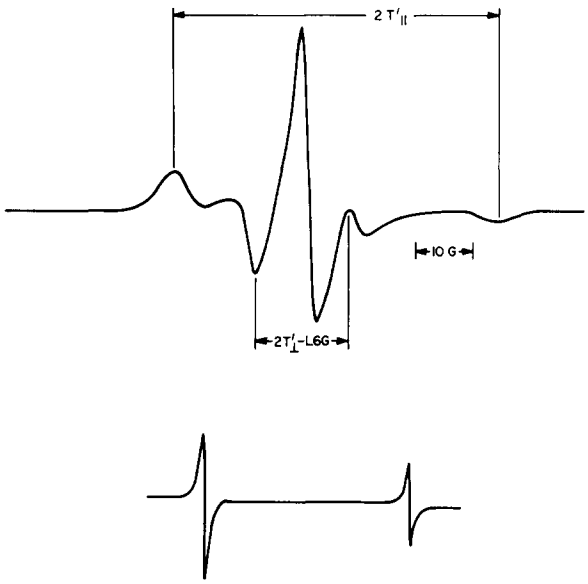

Fig 2 Typical ESR spectra of 5-NS (top)- and [^{15}N]Tempamine-d_{17} (bottom)-labeled erythrocyte ghosts Relevant spectral parameters from which the order parameter is calculated from the spectra of 5-NS are indicated In the case of the spectrum of [^{15}N]Tempamine-d_{17}, the rotational correlation time is calculated by well established equations [19–22] employing the single crystal anisotropic T-tensor values of 2,2,6,6-tetramethyl-4-maleimido[^{15}N]piperidine-1-oxyl-d_{17} [56] Instrument settings 5-NS, 100 G scan width, 0 32 G modulation amplitude, and 14 mW power, [^{15}N]Tempamine-d_{17}, 40 G scan width, 0 32 G modulation amplitude and 12 mW power

meric spectrin [32] Further, we wished to determine if the physical state of other membrane components, viz , bilayer lipids or cell surface sialic acid, depended on the state of aggregation of spectrin

Spectrin was isolated from spin-labeled erythrocyte ghosts and subsequently forced into the tetrameric or dimeric states of aggregation as described in Methods and confirmed by non-reducing, non SDS-PAGE Comparison of the W/S ratio ($W/S_{\text{tetrameric}} = 2\,34$, $W/S_{\text{dimeric}} = 2\,03$) showed that this parameter is much lower for isolated spectrin than in the entire membrane even though spectrin is the predominant protein labeled by MAL-6 in ghosts [26] This lower value of the W/S ratio of MAL-6 in isolated spectrin suggests that the conformation of this protein may be different isolated from the membrane than on the membrane, presumably because of the presence of other skeletal protein interactions of spectrin in the latter environment This suggestion is further supported by the ESR results of isolated spectrin in the tetrameric and dimeric states In contrast to the case of spectrin on the membrane, dimeric, isolated spectrin has a lower W/S ratio in the experiment described above than has tetrameric isolated spectrin This finding suggests that the increased W/S ratio of MAL-6 in erythrocyte membranes with increased amounts of dimeric spectrin (Table I) is not due to spectrin itself but perhaps to the altered associations between spectrin and other skeletal and bilayer proteins

This interpretation was strengthened by the results of the effects of 2,3-bisphosphoglycerate on the conformation of membrane proteins Sheetz [5] has shown that 2,3-bisphosphoglycerate destabilized the skeletal network of proteins isolated as a shell by the non-ionic detergent, Triton X-100 Upon addition of pH 8 0 solutions of 2,3-bisphosphoglycerate at various concentrations to spin-labeled membranes produced by lysis at 4°C from intact cells (and thus had little dimeric spectrin), the W/S ratio of MAL-6 was significantly elevated ($0\,001 < P < 0\,01$) over controls of pH 8 0 Na_2HPO_4 solutions of similar ionic strength and osmolarity (Table II) It is interesting to note that there was only a negligible increase in dimeric spectrin produced by 2,3-bisphosphoglycerate (data not shown), but that the W/S ratio was increased by about the same percentage as ghosts in which the amount of dimeric spectrin was con-

TABLE II

EFFECTS OF 2,3-BISPHOSPHOGLYCERATE ON THE PHYSICAL STATE OF MEMBRANE PROTEINS IN HUMAN ERYTHROCYTE GHOSTS AS MONITORED BY THE PROTEIN-SPECIFIC SPIN LABEL MAL-6

The W/S ratios are presented as means ± S D $N = 5$ except for 1 0 mM 2,3-bisphosphoglycerate (2,3-BPG) where $N = 4$ P values are calculated by a two-tailed Student's t-test

2 3-BPG (mM)	W/S ratio (% of control)	P
0	100	
1 0	116 ± 4 7	< 0 01
2 0	127 ± 5 9	< 0 001
4 0	133 ± 9 4	< 0 005

TABLE III

EFFECT OF SPERMINE ON THE PHYSICAL STATE OF MEMBRANE PROTEINS IN HUMAN ERYTHROCYTES AS MONITORED BY THE W/S RATIO OF THE PROTEIN SPECIFIC SPIN LABEL, MAL-6

The W/S ratios are presented as means ± S E for $N = 4$ samples for each concentration of spermine P values are calculated by a two-tailed Student's t-test

Spermine (mM)	W/S ratio (% of control)	P
0 0	100	
0 6	58 5 ± 4 2	< 0 005
1 2	54 9 ± 5 0	< 0 005
1 8	51 2 ± 5 4	< 0 005

siderably increased Since 2,3-bisphosphoglycerate is reported to decrease skeletal protein interactions [5], these results support the idea that increased segmental motion of spin-labeled skeletal proteins in erythrocyte membranes upon increased formation of spectrin dimers is a consequence of decreased skeletal protein-protein interactions

The polyamine, spermine, is reported to prevent the lateral diffusion of transmembrane glycoproteins [5] and to increase skeletal protein-protein interactions as determined by considerably higher shear forces necessary to disrupt and distend erythrocyte membranes treated with spermine [37] If the W/S ratio of MAL-6 were reflective of the protein-protein interactions of the erythrocyte membrane skeleton, then one would predict that, in contrast to the increased value of this ESR parameter upon disruption of protein-protein interactions caused either by an increased proportion of dimeric spectrin on the membrane (Table I) or 2,3-bisphosphoglycerate (Table II), a decreased value of the W/S ratio should result reflective of decreased segmental motion of membrane protein spin-label binding sites As shown in Table III the approx 40–50% decreased W/S ratio ($P < 0\,005$) shows that this prediction is completely borne out, again suggesting that the W/S ratio of MAL-6 accurately reflects the state of protein-protein interactions in the membrane

We presumed that since spermine is a polycation while spectrin is rich in negative charges [38], that an electrostatic interaction between spermine and the membrane was operative To test this presumption, ghosts spin labeled with MAL-6 were treated with 1 8 mM spermine and divided into two aliquots One aliquot was washed twice with the low ionic strength buffer, 5P8, the W/S ratio was changed only slightly (less than 5%) from the low value in ghosts that had been incubated with spermine but not washed with 5P8 (data not shown) The other aliquot of spermine-treated, spin labeled ghosts was washed twice with the relatively high ionic strength buffer, phosphate-buffered saline (pH 8 0), and twice with 5P8, the effect of spermine was almost completely abrogated – only a 4 4% decrease in the W/S ratio

TABLE IV

EFFECTS OF THE STATE OF AGGREGATION OF SPECTRIN AND 2,3-BISPHOSPHOGLYCERATE ON THE PHYSICAL STATE OF BILAYER LIPIDS AND CELL SURFACE SIALIC ACID

	Mean ± S D (number of samples) (% of control)			
	Spectrin state		2 3-Bisphosphoglycerate [a]	
	tetrameric spectrin	increased dimeric spectrin	1 2 mM	4 0 mM
S [b]	100 ± 0 6(2)	100 ± 0 3(2)	–	99 1 ± 0 4(4)
τ [c]	100 ± 5 5(3)	100 ± 6 8(3)	109 ± 3 7(3)	–

[a] Each 2,3-bisphosphoglycerate and control solution was adjusted to pH 8 0 The Na_2HPO_4 concentration was 5-times the 2,3-bisphosphoglycerate concentration to control for ionic strength

[b] The order parameter S is related to the average relative orientation of 5-NS in the lipid bilayer

[c] Calculated as described in Refs 19–22 employing the single crystal T-tensor values of 2,2,6,6-tetramethyl-4-maleimido[^{15}N]piperidine-1-oxyl-d_{17} [56]

relative to control spin labeled ghosts in 5P8 not treated with spermine ($P < 0.1$, $N = 3$) was found These results indicate that the spermine-protein interaction is essentially reversible and electrostatic in nature

In order to examine the effect of decreased skeletal protein interactions produced by either increasing the amount of dimeric spectrin on the membrane or by adding 2,3-bisphosphoglycerate, or the effect of increased skeletal protein interactions produced by spermine on the physical state of bilayer lipids and cell-surface sialic acid, 5-NS and perdeuterated, [^{15}N]Tempamine, respectively, were employed The average relative orientation of phospholipids (as discerned by the order parameter of 5-NS calculated from the experimental parameters indicated in Fig 2 [18,23]) was not altered by disruption of prtoein-protein interactions of the skeletal network by either of these two methods (Table IV) In contradistinction, the order parameter of 5-NS was slightly, but significantly, increased by spermine (Table V), implying an enhanced ordering effect of this polyamine on lipid orientation In order to determine if this effect in the membrane lipids were due to a direct interaction of spermine on membrane lipids or due to a secondary interaction on the membrane skeleton, spermine was added to spectrin-depleted ghosts or isolated lipids A slight increase in the order parameter of 5 NS in both spectrin-depleted ghosts (mean ± S D ($N = 4$) control, 0 693 ± 0 003, spermine, 0 703 ± 0 003, $P < 0.005$) and isolated lipids (mean ± S D ($N = 4$) control, 0 672 ± 0 003, spermine, 0 685 ± 0 004, $P < 0.005$) to which 1 2 mM spermine in buffer was added was observed These results suggest that spermine has only a slight effect on the relative average order of membrane lipids

The perdeuterated, [^{15}N]Tempamine spin label was used to monitor the physical state of cell surface sialic acid (70% of which is on glycophorin A [39]) in response to increasing the amount of dimeric spectrin and to the addition of 2,3-bisphosphoglycerate or spermine A typical spectrum of [^{15}N]Tempamine-d_{17} selectively attached to sialic acid by reductive amination is shown in Fig 2 (bottom) This spectrum is characterized by an apparent rotational correlation time, τ, which can be conceptualized as the time necessary for spin-labeled sialic acid to rotate through an angle of one radian [19–22] Neither method to disrupt skeletal protein-protein interactions caused a change in τ relative to the appropriate control (Table IV) In contrast, spermine was found to increase the motion of sialic acid by nearly 40% (mean ± S E $\tau_{spermine}/\tau_{control} = 0.611 \pm 0.014$, $N = 3$, $P < 0.002$)

TABLE V

EFFECT OF SPERMINE ON THE ORDER PARAMETER (S) OF 5-NS IN ERYTHROCYTE MEMBRANES

Spermine (mM)	S [a]	N	P [b]
0 0	0 694 ± 0 005	11	
0 6	0 701 ± 0 006	5	< 0 05
1 2	0 704 ± 0 004	5	< 0 002
1 8	0 707 ± 0 007	5	< 0 001

[a] Means ± S D are presented

[b] P values calculated by a two-tailed Student's t-test

Discussion

As noted in the Introduction, the erythrocyte membrane skeleton plays several crucial roles in the cell The physical state of skeletal proteins would seem to be important in these functions Palek and co-workers [14,16,40,41] and others [3,15,42] have described several hemolytic anemias in which erythrocyte membrane skeletal protein-protein interactions are altered In hereditary pyropoikilocytosis, an increased amount of dimeric spectrin, decreased skeletal stability, increased ease of thermal denaturation of spectrin, partial deficiency in the amount of spectrin, and of course altered cell shape are seen [14,16,40,41] In type I hereditary spherocytosis, a defective spectrin-band 4 1 association is postulated [15] In order to further understand the molecular interactions of these conditions and others and to learn more in general of the interactions of the skeletal network of proteins in erythrocytes, physicochemical methods such as spin labeling may play a role

In this study, the influence of the state of aggregation of spectrin and consequent skeletal protein-protein interctions on the physical state of membrane proteins, bilayer lipids, and cell surface

carbohydrates has been investigated Upon formation of increased amounts of dimeric spectrin on the membrane, an increased segmental motion of spin-labeled proteins (mostly located in the skeletal network [23–26]) is observed (Table I) A similar increase in motion was found by addition of 2,3-bisphosphoglycerate known to destabilize skeletal protein-protein interactions [5] (Table II) suggesting that increased dimeric spectrin on the membrane results in decreased skeletal protein interactions The manipulation of the state of aggregation of spectrin was shown to be reversible by non-reducing, non-SDS gels and by ESR (Table I) Spermine, known to increase protein-protein interactions [5,36], caused the W/S ratio of MAL-6 to be greatly decreased (Table III) These findings suggest that the MAL-6 ESR spectra of human erythrocyte membranes accurately reflect the molecular interactions in the membrane skeleton

Spectrin dimeric self-association to form tetramers involves SH groups [43] Extensive labeling of spectrin SH groups by *N*-ethylmaleimide resulted in increased amounts of spectrin dimer in the skeletal network of proteins [43], consequently, one needs to be certain that the maleimide spin label is not artifactually preturbing the spectrin dimer-tetramer equilibrium It is known that only 3–4 SH residues per spectrin dimer are spin labeled by MAL-6 [44], a sufficiently small number that the amount of dimeric spectrin was found not to increase when examined by non-reducing, non-SDS-gels [45] One might expect that if the spin label were not interfering in the self-association of spectrin dimers, then both the gel pattern and the W/S ratio of MAL-6 should be reversible for spin-labeled ghosts cyclically transformed from spectrin tetramer to dimer and back to tetramer These expectations were found (Ref 45 and Table I)

There is evidence that spectrin on the membrane and isolated spectrin do not behave similarly Liu and Palek previously noted that spectrin behaved kinetically different in the different environments [32] Our results support this evidence The W/S ratio of MAL-6 is significantly increased for dimeric spectrin on the membrane but in isolated dimeric spectrin, no increase in this parameter was found compared to tetrameric isolated spectrin (if anything, W/S was decreased) These differences undoubtedly reflect the influence of the interaction of spectrin with other skeletal proteins and band 2 1 The main point is that in at least some cases, inferences of the nature of spectrin on the membrane from studies of isolated spectrin should be tempered by these considerations

The presence of spectrin and the state of the skeletal protein network is reported to be involved in the maintenance of phospholipid asymmetry in erythrocytes [46] There are reports that in vitro band 4 1 and spectrin can bind to phosphatidylserine vesicles [12,13] Further, the in vitro association of band 4 1 with glycophorin A is reported to depend on triphosphoinositides [11] Although other interpretations are possible, the results presented here suggest that the order and motion of phospholipids may not be dependent on the state of spectrin aggregation (Table IV) If this interpretation is correct, these findings would be consistent with the idea that even though isolated membrane skeletal proteins may interact with lipids in vitro, such interactions between the phospholipids in the inner bilayer leaflet and the skeletal network of proteins, if present in membranes in situ, are not strong

The spatial orientation of the major sialoglycoprotein, glycophorin A, with respect to the membrane skeleton has been the subject of intensive research Biophysical and biochemical evidence for and against a direct linkage between glycophorin A and the membrane skeleton has been published [11,20,47–50] and reviewed in [29,30,46] The results of the present studies which show that decreased skeletal protein interactions resulting from dimeric spectrin or 2,3-bisphosphoglycerate do not affect the physical state of sialic acid may reflect that either glycophorin A and the skeletal network are not linked in situ, or that this glycoprotein is linked but any change induced in its physical state at the point of contact with the skeleton is not transferred to the cell-surface sialic acid Estimates of the number of copies of glycophorin A and band 4 1 indicate that there is a several-fold excess of the former protein [1] It is conceivable, as is the case of band 3 binding to band 2 1 [1], that only a fraction of glycophorin A polypeptides are bound to band 4 1 and that a weakening of the interactions between

this fraction of glycophorin A molecules and the skeleton would not be reflected in the motion of sialic acid because of the background of a much larger fraction of unbound molecules That an alteration in the skeletal network can result in changes in the physical state of sialic acid is exemplified by a finding of this study that spermine, a positively charged polyamine that increases skeletal protein interactions with the bilayer proteins, resulted in a 40% increase in rotational motion of this cell-surface carbohydrate, a trend also induced by the membrane protein crosslinking agent glutaraldehyde [51] The model to explain the spatial relationship of glycophorin A to the skeletal network which emerges from such studies is consistent with that previously proposed [5] in which the cytoplasmic pole of all or a majority of this transmembrane glycoprotein protrudes through interstitial holes of the skeletal meshwork Under certain conditions, the hole size of the meshwork can decrease resulting in an increased interaction between the proteins of the skeletal network and glycophorin A

Skeletal protein-transmembrane protein interactions in response to a binding event on the external side of the transmembrane protein are known (e g , phosphorylation of membrane proteins in response to adenylate cyclase induction by dopamine [52]) or proposed (e g , the role of the spectrin-like protein, fodrin, in the processes of memory in nerve cells [53–55]) as important physiological mechanisms

Continued investigation of protein-protein interactions of the skeletal network and between the skeletal network and transmembrane proteins in erythrocytes and other membrane systems will likely aid in understanding basic structural relationships in membranes and how these relationships are involved in physiological mechanisms and pathological conditions

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