Segmental Motions of Oxidized Human Erythrocyte Spectrin-Actin: A Spin Label EPR Study

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SEGMENTAL MOTIONS OF OXIDIZED HUMAN ERYTHROCYTE SPECTRIN-ACTIN
- A SPIN LABEL EPR STUDY

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

BÝ
BENITO O. KALAW

CHICAGO, ILLINOIS
MAY 1994
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To Jane and Ted
I want to extend my humblest thanks to all those who participated in the process which finally culminated in the writing of this dissertation:

Dr. Leslie Fung for the conception and support of this project, the training and professional guidance, the invaluable time and attention, many meaningful discussions, commenting on parts of dissertation section drafts, helping me write a manuscript for publication and for making me responsible for my own scientific growth and learning,

The Dissertation Committee Members for their willingness to serve on my committee and for giving invaluable suggestions throughout the completion of this work,

The Chemistry Department and Staff, the Graduate School, and Loyola University of Chicago for the financial support and the good learning environment,

Dean Francis Catania, Dr. JoAnn Rapp, Dr. James Rocks, Ms. Patty Robertson, and Ms. Diane Shaw for their encouragement, professional assistance and advice in successfully meeting my degree requirements,

Dr. Kenneth Olsen for the use of the Phast System Electrophoresis, Dr. Albert Rotermund, Jr. for the meaningful discussions about biochemical concepts and effective teaching strategies,

Dr. Albert Herlinger for his support, many valuable discussions, sensitivity and commitment to my well-being as a graduate student. He is a model educator who is interested in the unique development of students. His tips on effective teaching has helped me professionally. I also thank him for providing me with the desk and office space in his laboratory.
My thanks to Dr. Chakravarti Narasimhan and Dr. Helen Lu for the training on membrane and spectrin purification, Dr. Charles Thompson for the use of HPLC and the training in Organic synthesis in early part of my research, Michel Nehme for setting up the EPR program and Rita Hatfield for the use of computers in our laboratory.

While writing my dissertation, many others were helpful in many ways such as using computers, making graphs, drawing molecular structures, giving examples from their own experience, or discussing my results, ideas and procedures with me. I thank all the members of our research group, graduate students, faculty and friends for such help.

My communication with my adviser had been in a long distance way. I thank Bucknell University and Dr. Steen Pedersen for the use of computers, telephone and fax facilities.

Certainly not to be overlooked are my friends Hovis and Rene Imade, Brenda Stockwell, Elias Fernandez and Eugene Zaluzec - their collective help and encouragement made my seemingly impossible schedule survivable.

I am indebted to my mother and Aunt Florie for instilling in me the value of hard work, patience, stick-to-it-ness attitude, and my brother Jun for the financial support.

I thank my parents-in-law, Robert and Dorothy Cecil, for their love, encouragement of my curiosity, helpful advice, and the financial support during my graduate studies.

I have always felt extraordinarily blessed to have a loving, infinitely patient, attentive and understanding wife, Jane. She and my son, Ted helped me face the obstacles of graduate studies, and provided me with positive alternatives and the sense of possibility that I needed. To my wife and son, thank you for everything.
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<tr>
<td>$A_{280}$</td>
<td>optical absorption at 280 nanometers</td>
</tr>
<tr>
<td>Bis</td>
<td>N,N' -methylene-bis-acrylamide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DI</td>
<td>diamide</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
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<tr>
<td>$f_b$</td>
<td>bound fraction</td>
</tr>
<tr>
<td>GA</td>
<td>glutaraldehyde</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>$K^2$</td>
<td>scattering vector</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>Mal-6</td>
<td>4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy free radical</td>
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<tr>
<td>MW</td>
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</table>
nm
NMR
PAGE
0.3P7.6
5P7.4
PBS7.4
PH
pI
psi
RBC
s
S
SA
SA-DI
SA-GA
SA-H$_2$O$_2$
SA-PH
SDS
sec
SH
ST
TMEDA
TRIS

nanometers
nuclear magnetic resonance
polyacrylamide gel electrophoresis
0.3 mM sodium phosphate buffer, pH 7.6
5 mM sodium phosphate buffer, pH 7.4
phosphate buffer saline, 150 mM NaCl in 5 mM sodium phosphate pH 7.4
phenylhydrazine
isoelectric pH
pounds per square inch
red blood cell
strongly immobilized motion
signal amplitude of the low field region of s
spectrin-actin
SA treated with DI
SA treated with GA
SA treated with H$_2$O$_2$
SA treated with PH
sodium dodecyl sulfate
second
sulfhydryl
saturation transfer
N, N, N’, N’-tetramethylethylenediamine
tris [hydroxymethyl] aminomethane
UV  ultraviolet
w  weakly immobilized motion
W  signal amplitude of the low field region of w
$e^{1w}$  extinction coefficient
$e_{280}$  extinction coefficient at 280 nanometers
$\lambda_{\text{max}}$  wavelength at maximum absorption
$\tau_R$  rotational correlation time
$\tau_c$  rotational correlation time at the center field region of the ST EPR spectrum
$\tau_H$  rotational correlation time at the high field region of the ST EPR spectrum
$\tau_L$  rotational correlation time at the low field region of the ST EPR spectrum
$\mu g$  microgram
$\mu l$  microliter
CHAPTER 1

INTRODUCTION

1-a. Human erythrocyte oxidation

Oxidation of human erythrocyte often occurs as a consequence of the toxic action of various chemicals and drugs or their metabolites [1,2]. Oxidants can alter erythrocyte properties and cause hemolysis [3, 4]. The mechanisms for oxidant damage leading to altered erythrocyte membrane properties and hemolysis are not well understood, although some mechanisms have been proposed [5, 6]. In these mechanisms, erythrocyte damage is thought to be the end result of two processes: (1) the oxidation of hemoglobin followed by the denaturation of methemoglobin to hemichromes, and (2) the free radical attack on the membrane components such as the polyunsaturated fatty acid side chains of the membrane lipids, the reduced thiol groups and other susceptible amino acid side chains of the membrane proteins. The major targets of physiological oxidations are hemoglobin [7, 8], lipids [9-11] and membrane proteins [12, 13]. Of these, the proteins are more significant targets of oxidative injury than lipids [1].

The absence of a nucleus in the human red blood cell (RBC) makes protein modifications a major metabolic challenge for the cell [14]. There is no protein synthesis in the RBC, hence no replacement of damaged macromolecules. That makes RBC a good model for studying oxidative modifications and membrane damage [15] in more complicated cells of the brain [16, 17], heart [18], kidney [19-21], lymph nodes [22], intestine [23], other organ systems [24, 25]
and nonvertebrates [26-31].

1-b. Oxidation of erythrocyte membrane skeletal proteins

The human erythrocyte has a two-dimensional network of associated proteins that underlies the cytoplasmic surface of the plasma membrane now known as the membrane skeleton. This membrane skeleton exhibits complex material behavior and structural organization [32]. Recent understanding of the organization of the erythrocyte membrane skeleton has been spurred in large part by the application of molecular biology techniques [33].

Spectrin-actin (SA) is the basic structural component of the membrane skeleton network in the RBC [34, 35]. The complex consists of an irregular array of spider-like complexes with five [36] to six [37-39] spectrin per actin core. Each junction is stabilized by the formation of a ternary complex with protein 4.1 [40]. SA interactions that are independently weak are greatly stabilized by protein 4.1 and this stabilization occurs through direct interaction with spectrin. The SA network is highly spread out with varying degrees of extensional rigidity [41].

SA is key to the regulation of membrane deformability and mechanical stability [42-45]. For example, it is known that extraction of spectrin results in membrane fragmentation and generation of spherical fragments [46]. Heating of biconcave RBCs to the temperature at which spectrin denatures causes membrane budding and fragmentation with decoupling of the lipid bilayer from the underlying network [47]. Deficiency in spectrin in human RBCs results in spherical morphology [48]. All these studies suggest the deformability and stability roles for spectrin. SA also provides structural support to the bilayer interface [45, 49], a support that is essential for the prolonged survival of RBCs in the high shear environment. The RBCs constantly change shape as they are subjected to a range of fluid forces during circulation. In the capillaries, the RBCs folds along a longitudinal axis, assume an asymmetric shape with unique
flexibility and elasticity that is maintained throughout their passage through the capillaries [44]. SA anchors and distributes transmembrane proteins that regulate ion transport and membrane surface charge [50].

Spectrin is the major membrane skeletal protein. It is composed of two non-identical units subunits (α spectrin and β spectrin) intertwined side to side in an antiparallel manner to form a heterodimer. Spectrin heterodimers associate to form tetramers, and this species appear to predominate in the membrane skeleton [51]. An increase in intramolecular association or intermolecular associations of the membrane skeletal proteins, or an increased association of the cytoplasmic domains of transmembrane proteins with the spectrin-actin network will markedly inhibit the ability of the membrane to undergo deformation by limiting the ability of spectrin to rearrange [41].

Increased membrane permeability and leakiness and decreased deformability were observed in RBCs treated with diamide (DI), phenylhydrazine (PH) and hydrogen peroxide (H₂O₂) [43, 52-59]. Among the membrane components modified by these oxidants, spectrin, with exposed SH groups, was found to be oxidized via intra- and interchain disulfide bonds to give high molecular weight crosslinked products [52-55, 56-67]. Spectrin crosslink aggregates were also found in RBCs that were from Vitamin E-deficient [11], glucose-6-phosphate dehydrogenase deficient [68-70], pyruvate kinase-deficient [70] or diabetic individuals [71]. Complexes of spectrin and hemoglobin exist in senescent RBCs, β-thalassemic, sickle and xerocytic RBCs [72, 73]. In view of these findings, crosslinked spectrin aggregates can be used as an index of oxidative damage.

Fung and Johnson [74] have shown that spectrin exhibits considerable segmental motions. Early spin label electron paramagnetic resonance (EPR) studies showed that saturation transfer detection, with a time scale of 10⁴ to 10³ s, was sensitive to the dynamics of spectrin, and that
the molecular motions were unrestricted by other membrane components [74]. Studies with EPR at X-band and Q-band indicated that spectrin exhibits multiple classes or rates of motion. The three principal motional components appear to have correlation times of $\leq 10^{-9}$, $10^{-7}$ to $10^{-6}$, and about $10^{-3}$ s, and have different sensitivity to pH and temperature changes [74]. Other EPR studies showed that spin labeled spectrin dimers and tetramers exhibit similar rotational dynamics in solution, in the $10^{-6}$ s range at 2 °C [75, 76]. These results also confirmed that the dynamics of spectrin remain the same in samples associated with membrane-bound ankyrin. Nuclear magnetic resonance (NMR) studies also confirm the existence of rapid segmental motions ($10^{-10}$ - $10^{-9}$ s) as well as relatively slow motions (slower than $10^{-6}$ s) in spectrin [77, 78]. Furthermore, using one pulse and spin echo NMR techniques, we have shown that the fraction of protons in rapid motion is about 15 % of the total protons in the spectrin molecule at 37 °C in phosphate buffer with 150 mM NaCl at pH 7.4 [78]. These findings are supported by results obtained by other physical methods. Phosphorescence anisotropy studies reveal spectrin dimer correlation times of $3 \times 10^{-6}$ s and spectrin tetramer correlation times of 3 and $30 \times 10^{-6}$ s at 10 °C [79], whereas transient dichroism studies gave correlation times of about $10^{-4}$ s at 4 °C [80]. We have recently extended this work to dynamic light scattering (DLS) measurements on spectrin over a temperature range from 23 to 41 °C as a function of the square of the scattering vector ($K^2$) over the range of $0.7 \leq K^2 \leq 20 \times 10^{10}$ cm$^{-2}$ [81]. Analysis of the autocorrelation functions collected for these solutions reveals the presence of two predominant motional components over the full $K^2$ range. The behaviors of the fast and slow components as a function of $K^2$ were interpreted in terms of protein motions by modeling spectrin heterodimers and tetramers as wormlike coils. In the low $K^2$ region the slow ($7.3 \pm 2.0 \times 10^{-8}$ cm$^2$/s) and fast ($20.3 \pm 2.0 \times 10^{-8}$ cm$^2$/s) components were assigned to the center of mass diffusion coefficients for tetramers and dimers, respectively. The magnitude of diffusion coefficients at 20 °C of the fast
component \((39.4 \pm 2.0 \times 10^{-4} \text{ cm}^2/\text{s})\) suggested the emergence of much faster motions, surmised to be subglobal segmental motions of spectrin tetramers. These measurements supply further evidence for fluctuational segmental motions of spectrin occurring over a relatively large distance of 22 to 30 nm with relaxation times \(\leq 23 \times 10^{-6} \text{ s}\).

1-c. Statement of the problem

It is tempting to assume that oxidation of SA in membranes render immobilized SA molecules to give rigid membrane skeleton and thus, cells with reduced deformability. However, no correlation between membrane rigidity and SA segmental motions has been reported. It is not clear whether the cross-linking of SA produces SA molecules with restricted mobility.

To explore the role of oxidation on the conformational flexibility and dynamics of SA, we asked how and to what extent the degree of crosslinking affects the segmental motions of SA using conventional and saturation transfer (ST) EPR. Ultimately, we want to find out how these oxidation effects are related to molecular/cellular event in the RBC like membrane rigidity.

Over the past years, spin label EPR experiments has proved essential in providing insights into a range of biochemical and biophysical findings. Structural and motional dynamics information obtained from these experiments can provide new perspectives on the molecular mechanisms of cell rigidity, RBC lysis and aging.
CHAPTER 2

MATERIALS AND METHODS

2-a. Chemicals

N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (Mal-6) was purchased from Aldrich (Milwaukee, WI); agarose, acrylamide, ammonium persulfate, bis-acrylamide, Coomassie Blue 250, N,N,N’,N’-tetraethylmethylenediamine (TMEDA), pyronin and sodium dodecyl sulfate from Biorad (Richmond, CA); silicone liquid (200 fluid) from Dow Corning Corporation (Midland, MI); acetonitrile, copper sulfate (CuSO₄·5 H₂O), hydrochloric acid (HCl), sodium phosphate (NaH₂PO₄ and Na₂HPO₄), potassium ferricyanide, sodium carbonate (Na₂CO₃), sodium chloride (NaCl), sodium hydroxide (NaOH) and sodium tartrate from Fisher Scientific (Pittsburg, PA); Phastgel (SDS) from Pharmacia (Piscataway, NJ); protein assay reagent from Pierce (Rockford, IL); β-galactosidase, bovine serum albumin, carbonic anhydrase, diazinedicarboxylic acid bis[N,N-dimethylamide] (DI), dithiothreitol (DTT), egg albumin, glutaraldehyde (GA, Grade I, 25% aqueous solution), hydrogen peroxide (H₂O₂), myosin, phenylhydrazine (PH), phosphorylase b and were purchased from Sigma Chemical Co. (St. Louis, MO).

2-b. Modification of spin labeled spectrin-actin by glutaraldehyde (GA), diamide (DI), hydrogen peroxide (H₂O₂) and phenylhydrazine (PH)

2-b.1. Preparation of RBC membranes

Human blood samples in CPDA-1 or ADSOL anticoagulant were obtained from a local blood banks (Red Cross/Life Source). About 5 ml of the packed cells were gently mixed with
35 ml of cold phosphate buffer saline (PBS, 150 mM NaCl in 5 mM sodium phosphate buffer at pH 7.4) and centrifuged at 4,000 rpm (about 2,000 g) with a Sorvall RT6000B rotor for 4 minutes, 4 °C. After centrifugation, the buffy coat and the supernatant were carefully aspirated and discarded. This step was repeated two more times to give washed RBCs.

Membranes were prepared from the washed RBCs by hypotonic lysis using 5 mM phosphate buffer pH 7.4 (5P7.4)[82]. In this procedure, washed RBCs in 5P7.4 were centrifuged at 16,000 rpm (31,000 g) using a Sorvall RC-5B SS-34 rotor for about 8 minutes at 4 °C. The supernatant and the proteases-containing granulocyte button were very carefully aspirated and discarded. The procedure was repeated five or 6 more times to get the membranes. The absorbance of the membrane at 280 nm (A_{280}) was obtained and converted to membrane protein concentrations using a calibration curve established in this laboratory. The Lowry Assay [83] and as modified by Peterson [84] was used to determine the protein concentration in establishing the calibration curves.

2-b.2. Spin labeling of RBC membranes

Membrane samples (usually about 4 mg/ml in protein concentration) were spin labeled with the protein spin label N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (Mal-6). In this procedure, an aliquot of the spin label stored in acetonitrile, was added to a vial, and the acetonitrile was evaporated under N\textsubscript{2} gas before the addition of the membrane sample (30-50 µg Mal-6 per milligram of protein) in the dark at 4 °C for 1 hr [85]. Excess spin label was removed by washing with 5P7.4 buffer three times at 4 °C until the samples gave constant EPR signals. A portion of labeled membranes was set aside for crosslinking studies.
2-b.3. Modifications of spin labeled red cell ghosts by GA, DI, H₂O₂ and PH

Spin labeled membranes modified by GA

The spin labeled membranes in 5P7.4 were diluted with the same buffer to a concentration of about 2 mg/ml then treated with freshly prepared GA, and incubated at 22 °C for 20 minutes [62]. The final concentration of the membrane was about 1 mg/ml. (It is very important to check the purity of GA by scanning between 230 and 300 nm before use because pure GA absorbs at 280 nm). Membranes were freed from excess GA by washing three times with 5P7.4 at 32,000 x g for 8 minutes at 4 °C [80].

Spin labeled membranes modified by DI, H₂O₂ and PH

The spin labeled membranes in 5P7.4 (concentration about 2 mg/ml in protein) were treated with freshly prepared DI, H₂O₂ and PH in 5P7.4. The final concentration of the membrane in the mixture was kept at 1 mg/ml. The mixture was gently mixed and then incubated at 37 °C at appropriate times. The incubation times for the oxidized samples were as follows: 0.5 mM H₂O₂-treated sample, 15 min [59]; 1 mM DI-treated sample, 60 min [87] and 20 mM PH-treated sample, 60 min [65]. Excess oxidant was removed by washing three times with 5P7.4 at 32,000 x g for 8 minutes at 4 °C.

2-b.4. Preparation of SA from spin labeled membranes

Spin labeled SA was extracted from spin labeled membranes by low ionic extraction [82]. Spin labeled membranes were washed once with 0.3 mM phosphate buffer pH 7.6 (0.3P7.6) then diluted with the same buffer from a concentration of 4 mg/ml in protein to about 2 mg/ml. The mixture was incubated for 30 minutes at 37 °C to solubilize SA and centrifuged at 63,000 rpm (400,000 x g) with a Sorvall T865.1 rotor for 25 minutes at 4 °C. The SA in the supernatant was
carefully removed and concentrated in an ultrafiltration cell (Amicon) with a membrane that had a molecular weight (MW) cut-off of 100,000 (YM-100) at 4 °C under N₂ (at a pressure of 10 psi) to a final concentration of about 2 mg/ml followed by dialysis with desired buffer. The extinction coefficient (ε¹%) of 10 [89] was used to determine the concentration of SA samples. The concentrated spin labeled SA was kept at 4 °C until use.

2-b.5. Modification of spin labeled SA by GA, DI, H₂O₂ and PH

Spin labeled SA (about 2 mg/ml) in 5P7.4 was modified with various reagents in the same buffer under the following conditions: a) 40 mM GA for 20 minutes [62]; b) 2 mM DI for 60 minutes [87]; c) 40 mM PH for 60 minutes [65]; d) 0.5 mM H₂O₂ for 15 minutes [59]. After incubation, the modified samples (final concentration about 1 mg/ml) were dialyzed and concentrated to about 10 mg/ml by vacuum dialysis (Prodicon, Danvers, MA) using the appropriate buffer at 4 °C overnight, and then concentrated by low speed centrifugation when necessary at about 500 x g (2,000 rpm) using an SS-34 rotor at 4 °C.

2-c. Sample Analysis

2-c.1. Protein Assay

Protein concentrations of the modified SA were determined by the modified Lowry Assay [90]. This procedure is considerably more rapid and convenient, and incidentally a little more sensitive. In this procedure, BSA was the standard and the extinction coefficient of 0.667 for a solution of BSA at 1.00 mg/ml was used. To generate the calibration curve, 5 to 100 µg of protein (in 100 µl volume) was placed in each tube containing 1.0 ml of Lowry C reagent. (Lowry C reagent [83] was made by mixing 1 part of 0.5% (w/v) CuSO₄·5 H₂O with 50 parts of 2% Na₂CO₃, 0.1 N NaOH, 0.16% Na tartrate; CuSO₄·5 H₂O, Na₂CO₃, Na tartrate and phenol
reagent were all reagent grade and were used without prior purification). One hundred µl of Folin reagent (previously diluted 1:1 with water) was then added to each sample, followed immediately by vortexing. (Immediate, complete mixing of the Folin reagent with Lowry C solution was critical. The acidic Folin reagent was more dense than the basic Lowry solution. If not fully mixed, some fell to the bottom of the tube where both acidic pH and intact yellow phospho-molybdo-tungstate complex were maintained. Added DTT reduced the intact free complex that gave anomalous and high-absorbance values). After 3 minutes, 100 µl of 20 mM DTT was added to each reaction mixture, and vortexed again. The absorbance of the solution was measured at 740 nm. A plot of absorbance as a function of BSA concentration (or µg of BSA) was obtained, and from this plot, the concentration of the unknown protein was determined.

2-c.2. Electrophoresis

SDS-polyacrylamide gel electrophoresis (4 % acrylamide, either home made or from Phast System, Pharmacia, NJ) was carried out on the modified samples with DTT [62] on GA-treated SA (SA-GA) and without DTT [3] on DI-treated (SA-DI), H₂O₂-treated (SA-H₂O₂), and PH-treated (SA-PH) samples. In this procedure, the modified samples were warmed for 20 minutes at 37 °C. After warming the mixture of the SDS-concentrate and the modified SA samples, at least 40 µg was loaded on to the polyacrylamide gels. Subsequent electrophoresis were done using Phast System (Pharmacia, NJ) followed by staining with Coomassie Blue R250. (Initially, tube gels were used for electrophoresis instead of Phast Gel). A gel scanner (Model 1312, ISCO Research, IL) was used at 520 nm [91] to determine the mobility and intensity of the bands quantitatively. Molecular weight estimates were obtained from the log molecular weight versus mobility curve using the following standards: carbonic anhydrase (29,000); egg albumin (45,000); bovine serum albumin (66,000); phosphorylase b (97,400); Escherichia
coli β-galactosidase (116,000); and rabbit myosin (205,000). The MW of protein complexes with MW higher than 200,000 were estimated by extrapolating the calibration curve to about 700,000 daltons. The intensities of individual protein bands and the total intensities of all the protein bands for each sample in each lane were obtained from densitometer tracings and the percentage of the individual band intensity in the lane was calculated.

2-c.3. UV spectral analysis

In order to detect changes in the chromophoric groups accompanying reaction with DI, GA, H₂O₂ and PH, crosslinked or oxidized samples (about 1 mg/ml) in 5P7.4 were scanned from 230 to 330 nm and the maximum absorption measured using a DU-65 Beckman spectrophotometer. The buffer 5P7.4 was used as a blank.

2-c.4. pI studies

To determine the isoelectric pH (pI) of the spin labeled SA samples (about 1 mg/ml in protein concentration) after modification, the pH of the samples were adjusted with 1 N HCl at 22 °C [85]. The samples at different pH values were centrifuged at 2000 rpm (about 500 x g) with a Sorvall RT 6000B), and the absorption at 280 nm (A₂₈₀) of the supernatant was obtained, using 5P7.4 as blanks. The absorbance values were then converted to concentration values, which were plotted against the corresponding pH for the modified and unmodified samples. The pH values corresponding to the lowest concentrations in the figures were taken as pI point of the sample.

To prepare the EPR samples, 5-8 ml of sample (concentration about 1 mg/ml) was precipitated with HCl to its pI as above, and then centrifuged at 2000 rpm with a Sorvall RT 6000B for 4 minutes at 4 °C. The supernatant was carefully separated from the precipitate and
the $A_{280}$ of the supernatant was obtained. The samples were washed with appropriate buffer (pH of the wash buffer is the pI of the sample upon addition of HCl) at 2000 rpm with a Sorvall RT 6000B rotor for 4 minutes at 4 °C. The precipitate was washed three times until EPR signal was constant and EPR was carried out on the sample immediately.

2-d. EPR Measurements

2-d.1. Methods

Conventional EPR

EPR experiments were performed on E-109 Century line EPR spectrometer equipped with an E-102 microwave bridge, an ER4111 variable temperature unit (IBM Instruments Inc., Danbury, CT) and a V-7700 power supply (Varian Associates, Palo Alto, CA). The spectrometer was interfaced to a Zenith personal computer. EPR samples were introduced into 50 µl capillary tubes, following the procedures used in this laboratory. The sample tubes were centered by specially constructed holders. The spectra were recorded at 9 GHz. Conventional in-phase ($V_1$) absorption EPR spectra were recorded at non-saturating microwave power and a modulation amplitude of 1 gauss. The field sweep was 100 gauss. Quantitative measurements of spin label concentrations were performed by using the standard spectrometer calibrations: field modulation, 100 kHz; receiver gain, $1.25 \times 10^4$; time constant, 0.128; scan time, 60 seconds; microwave frequency, 8.95 GHz; incident microwave power, 1 mW. Since phenylhydrazine can reduce the nitroxide spin labels as shown by the low signal to noise ratio in the EPR spectra, it was necessary to add potassium ferricyanide (final concentration of 1 mM) to the spin labeled sample. Potassium ferricyanide addition regenerated the spin labels. Before the EPR measurements, the temperature was carefully equilibrated. The relative precision of individual temperature measurement was about ±0.5 °C with an overall accuracy of ±1 °C. Silicone fluid
was used to half-fill the quartz tube and a copper constantan thermocouple was inserted into the sample as a quick check to monitor the temperature of the sample before recording the spectra.

**Saturation transfer (ST) EPR**

ST-EPR spectra were recorded in second harmonic, 90° out-of-phase ($V''_2$), absorption mode. Standard spectrometer settings [86] were used: modulation amplitude, 5 gauss; time constant, 0.128; scan time, 60 seconds; microwave power, 42 mM. The phase settings were adjusted by the "self-null" method [87]. In this procedure, the field is centered on a major spectral line and the approximate "phase null" is located by minimizing the signal. The exact phase null is then determined by progressively measuring the signal height at 0.2 - 0.4° above and below the approximate phase null. Four measurements were generally made on each side. The signal amplitude was plotted against the phase setting and a straight line drawn through the points, with the zero intercept being used as the final phase setting for the spectrum. The determination of exact phase nulls is critical in ST-EPR spectra measurements because phase changes as small as 0.2° can produce measurable change in spectral shape. Care and consistency are a must in determining the exact phase nulls.

2-d.2. **EPR Data Analysis**

**Two component conventional EPR spectra**

The conventional EPR spectrum, $V_1$, of the spin labeled SA consists of two components: one set of narrow lines and one set of broad lines, with center overlapping each other (Figure 4). In the spectrum, the narrow lines were assigned to the nitroxides with weakly immobilized motions ($w$) and the broad lines were assigned to nitroxides with strongly immobilized motions ($s$) [92]. The signal amplitudes of the low field region of $w$ and $s$, $W$ and $S$ respectively, were
measured. The W/S ratios of SA without oxidant/crosslinking reagents were calculated. The compositions of w and s in each sample, assuming the sample consisted of only w and s motions, were also obtained using the relationship between the change in W/S versus the percent motional composition [94]. Briefly, a linear regression line relating the change in W/S with motional composition was obtained and the slope of the line was determined. Substituting the change in W/S due to crosslinking gave the motional composition (% w and % s) corresponding to each W/S ratio. Spectral subtraction to remove the fast motional component was also used [74].

To get additional quantitative information on the reaction of SA and the crosslinker (or oxidant), the W/S ratios of SA samples without crosslinker (or oxidant), (W/S)$_{sa}$, and of SA with a certain amount of crosslinker present, (W/S)$_{x}$, were measured. The W/S ratio corresponding to the completely crosslinked protein, (W/S)$_{sa-x}$, was then determined by non-linear regression method. The fraction of W/S ($f_b$) modified at each crosslinker concentration can be calculated from the expression:

$$ (W/S)_x = f_b (W/S)_{sa-x} + (1-f_b)(W/S)_{sa} $$

or:

In terms of the relationship between $f_b$ and change in W/S ratio,

$$ \Delta(W/S)_x = f_b \Delta(W/S)_{sa-x} $$

Here, it was assumed that the W/S ratios observed on addition of crosslinker to spectrin-actin were the direct results of the modifier reacting with SA to affect the spin label mobility.
**ST EPR Spectral Analysis**

The $V_2'$ spectrum was used to study the motions associated with the S signal. The spectrum consists of low, central and high field regions with amplitudes $L$, $L''$, $C$, $C'$, $H$, and $H''$ [95]. The amplitude ratios were measured and the signal amplitude ratios $L''/L$, $C'/C$, and $H''/H$ were calculated from the spectrum (before and after spectral subtraction) to give values of rotational correlation times [74, 95] that describe differing rates of motion in the slow motion time domain.

**Spectral Subtraction**

Spectral subtractions were carried out following the published procedure [71] and using the program ASYST. The second harmonic 90° out of phase, absorption spectra at pH 6 and pH 8 recorded at 20 °C were used. The removal of the weakly immobilized component was first done. The appropriate fraction of a pH 6 spectrum was subtracted from the spectrum at pH 8 to give the single-component spectrum of the fast motion. Appropriate fractions of this fast motion component were subtracted from the original spectra to yield the slow motion component spectra.

To get "normal" spectra from the subtractions, appropriate scaling factors that removed the fast components were chosen. Double minima, for example, result if the scaling factors used are larger than those used here. Therefore, consistency and care in the use of the scaling factors were followed to get reproducible results from this procedure. At X-band, the high-field lines of the fast component lie in a relatively "flat" region of the ST-EPR spectra, so accurate subtractions were achieved. Large signal to noise ratio (about 7) was necessary for acceptable subtraction.
CHAPTER 3

RESULTS

3-a. Extent of SA crosslinking by GA, DI, H₂O₂, and PH

The use of mild reactions were most suitable because excessive crosslinking produced high molecular weight (HMW) aggregates which failed to enter the gels. Dissolving the SA in SDS was the optimal procedure to stop the crosslinking reactions. No crosslinking was observed when SDS was added presumably because it rapidly abolished the native juxtaposition of the polypeptides. The decrease in intensity of spectrin (bands at 220 kD and 240 kD) and actin (band at 45 kD) and the increase of high molecular weight component intensity in the SDS-PAGE gels of samples modified by GA, DI, H₂O₂ or PH were used to measure the degree of SA crosslinking when GA, DI, H₂O₂, or PH were added.

Our results from the SDS-PAGE in the presence of DTT for SA-GA samples (Figure 1) show that the total intensity of spectrin and actin diminished progressively as the concentrations of GA increased from 0 to 20 mM. Only 19.4 ± 2.3 % (n=5) of the spectrin and actin band intensities remained and 80.6 ± 1.3 % (n=5) of the high molecular weight complexes appeared in SA-GA (20 mM) samples. The intensities of the bands assigned to high molecular weight complexes (HMW) at 440 ± 15 kD (n=5) and 650 ± 10 kD (n=5) increased with increasing GA concentrations. It is likely that these HMW complexes were multiple spectrin heterodimers. At 10 mM or higher GA concentration, the band intensity of the HMW complexes was about 80 %. It is interesting to note the disappearance of actin (Band 5 with MW of 45 kD) at GA concentration of 10 mM. At GA concentrations higher than 20 mM, most of the band intensities stayed on top of the gel indicating that large aggregates were formed, and therefore, the
Figure 1. The effect of glutaraldehyde crosslinking on human erythrocyte spectrin-actin. After incubation at pH 7.4 under conditions that maximize spectrin modification, SDS was added and electrophoresis carried out. The amount of protein in gel stained with Coomassie blue was expressed as % peak area as determined by quantitative densitometry at 520 nm. HCx = high molecular weight complex; Band 5 = actin; [GA] = concentration of glutaraldehyde (mM).
determination of the degree of crosslinking was difficult to assess.

The oxidation of SA by DI was also concentration-dependent. As shown by SDS-PAGE in the absence of DTT, about 34.6 ± 2.8 % for the spectrin and actin bands remained when the DI concentration in the incubation mixture reached 1 mM DI (Figure 2). Bands of HMW complexes of 463 ± 17 kD (n=4) appeared as DI concentration increased, and the band intensity reached 65.4 ± 2.8 % when the DI concentration was about 1 mM. The band intensity of Band 5 remained at about 10 % in the modified and unmodified samples, indicating that actin was not involved in the crosslinking in the samples treated with diamide. The modification of SA by DI was also temperature-dependent. At 0, 10, 20 and 37 °C at pH 7.4, the percentages of peak area of spectrin and actin were about 70, 50, 40 and 30 %, respectively (data not shown). The MW of the modified complex obtained at these temperatures were all about 464 ± 8 kD (n=4).

For SA-H$_2$O$_2$ samples, as shown by SDS-PAGE in the absence of DTT, about 44.4 ± 3.8 % of the intensities of spectrin and actin remained, while the band intensities of the HMW complexes at 457 ± 9 kD (n=3) reached a level of about 55.6 ± 4 % with H$_2$O$_2$ concentrations at 0.5 mM or higher (Figure 3). The intensity of Band 5 remained at a level of 10 % throughout the concentration range used indicating that actin was not involved in the crosslinking of the samples oxidized with H$_2$O$_2$.

Similar results were observed in SA-PH mixtures. The spectrin and actin intensities in SDS-PAGE in the absence of DTT decreased from about 90 % to about 28.4 ± 5 % (n=2) at a PH concentration of 20 mM (Figure 4). The intensities of the HMW complex at 450 ± 7 kD (n=2) reached 56.6 ± 1.4 % when the concentration of PH reached about 20 mM. The intensities of Band 5 remained at about 15 % throughout the PH concentration range used. Therefore, actin was not crosslinked to spectrin in the PH-treated SA samples.
Figure 2. The effect of diamide on spectrin-actin at pH 7.4 using SDS-PAGE. % peak area is the amount of protein in gel stained with Coomassie blue as determined by quantitative densitometry at 520 nm. Hcx = high molecular weight complex; Band 5 = actin; [DI] = concentration of diamide (mM).
Figure 3. The effect of hydrogen peroxide on spectrin-actin at pH 7.4 using SDS-PAGE. The % peak area is the amount of protein in gel stained with Coomassie blue as determined by quantitative densitometry at 520 nm. HCx = high molecular weight complex; Band 5 = actin; \([\text{H}_2\text{O}_2]\) = concentration of hydrogen peroxide (mM).
Figure 4. The effect of phenyl hydrazine on spectrin-actin at pH 7.4 using SDS-PAGE.
The % peak area is the amount of protein in gel stained with Coomassie blue as determined by quantitative densitometry at 520 nm. HCx = high molecular weight complex; Band 5 = actin; [PH] = concentration of phenyl hydrazine (mM).
Figure 5. Protein densitometric scan of spectrin-actin control at pH 7.4 and 20 °C electrophoresed on 4 % acrylamide gel. Gel scanning was carried out at 520 nm.
a) SA

b) SA-GA

c) SA-DI

d) SA-H₂O₂

e) SA-PH
**TABLE 1.**

The extent of crosslinking on spectrin-actin crosslinking by oxidants glutaraldehyde, diamide, H$_2$O$_2$ and phenylhydrazine.

<table>
<thead>
<tr>
<th>System</th>
<th>Incubation Condition</th>
<th>% spectrin-actin$^b$</th>
<th>pI$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-Buffer</td>
<td>37 °C, 60 min</td>
<td>0 (n=8)</td>
<td>4.47 ± 0.03 (n=3)</td>
</tr>
<tr>
<td>SA-GA (20 mM)</td>
<td>22 °C, 20 min</td>
<td>17.7 ± 2.3 (n=5)</td>
<td>4.81 ± 0.02 (n=3)</td>
</tr>
<tr>
<td>SA-DI (1 mM)</td>
<td>37 °C, 60 min.</td>
<td>34.6 ± 2.8 (n=4)</td>
<td>4.91 ± 0.08 (n=3)</td>
</tr>
<tr>
<td>H$_2$O$_2$ (0.50 mM)</td>
<td>37 °C, 60 min.</td>
<td>44.4 ± 3.8 (n=2)</td>
<td>4.84 ± 0.01 (n=2)</td>
</tr>
<tr>
<td>SA-PH (20 mM)</td>
<td>37 °C, 60 min.</td>
<td>28.0 ± 2.5 (n=2)</td>
<td>4.75 ± 0.03 (n=2)</td>
</tr>
</tbody>
</table>

$^a$The samples consisted of 1 mg/ml (about 2 µM) spin labeled spectrin-actin (SA) with or without the following chemicals: glutaraldehyde (GA), diamide (DI), hydrogen peroxide (H$_2$O$_2$), or phenylhydrazine (PH) in 5 mM phosphate buffer at pH 7.4.

$^b$The proportions of remaining spectrin-actin in samples with or without glutaraldehyde, diamide, H$_2$O$_2$ or phenylhydrazine were detected by 4 % SDS-PAGE, with densitometer tracings to indicate band intensities. Bands at 240 kD, 220 kD and 45 kD were considered as spectrin-actin.

$^c$The isoelectric pH (pI) of the spin labeled spectrin-actin and modified spectrin-actin samples (protein concentration of 1 mg/ml at pH 7.4) were obtained by adjusting the pH of the samples in the range of 4.2 to 5.2 with 1N HCl at 22 °C. The pH values corresponding to the lowest concentrations were taken as the pI of the sample. See text for details.
A summary of the data of SA crosslinking by GA, DI, H$_2$O$_2$ or PH is given in Table 1. Representative protein gels scans of SA control and modified SA samples are compared in Figure 5.

3-b. $U_{\text{max}}$ of Modified SA

One application of UV spectroscopy to proteins involves monitoring the perturbations of the chromophores (e.g. tryptophan, tyrosine, phenylalanine, etc.) that give rise to the absorption bands. Such perturbations can cause a change in the absorption spectra. One such type of perturbation is chemical modification. To see how the degree of crosslinking affects the optical absorption properties of SA, UV studies were performed. At pH 7.4, the UV absorption spectra of the SA modified with 1 mM DI, 20 mM GA, 0.5 mM H$_2$O$_2$, and 20 mM PH exhibited new maxima at 272 nm, 279 nm, 277 nm and 278 nm, respectively (Figure 6). Slight increases in absorbance relative to the control were also observed. Determination of the extinction coefficients ($\epsilon_{\text{max}}$) showed slight increases in $\epsilon_{\text{max}}$. The $\epsilon_{\text{max}}$ changed from 1.10 (SA) to 1.53 (GA), 1.20 (DI), 1.32 (H$_2$O$_2$), and 1.51 (PH), respectively.

3-c. $pI$ of modified SA

The change in pH is one perturbation that can be induced in proteins. Since pure SA is highly negative at physiological pH, determining the response of the modified SA to decreasing pH could give us information on the charge properties and aggregatability of the modified SA samples. To do these studies, the concentration of the soluble component in unmodified SA in 5 mM phosphate buffer at different pH values in the range of 4.2 to 4.6 was determined. The concentration of SA at pH 7.4 was 2 µM (1 mg/ml). As the pH of SA was adjusted to $\leq$ 5 at 22 °C, the solubility of SA decreased as shown by the increased precipitation, accompanied by
Figure 6. The UV spectra of modified spectrin-actin and control at pH 7.4. The protein concentrations of these samples were identical in the experiment. 1 = spectrin-actin; 2 = glutaraldehyde (20 mM); 3 = diamide (1 mM); 4 = hydrogen peroxide (0.5 mM); 5 = phenyl hydrazine (20 mM).
a decrease in $A_{280}$ of the soluble fraction. Decreases in solubility in differing extents were also observed in the crosslinked, spin labeled SA samples upon adjusting the pH in the range of 4.2 to 5.2. The average $\epsilon_{280}$ of the samples were found to be $1.098 \pm 0.071$ (SA), $1.492 \pm 0.008$ (SA-GA), $1.228 \pm 0.014$ (SA-DI) and $1.508 \pm 0.002$ (SA-PH). The isoelectric pH values were $4.47 \pm 0.03$ (n=3) for 2 $\mu$M SA, $4.75 \pm 0.03$ (n=2) for SA containing 20 mM PH, $4.81 \pm 0.01$ (n=3) for SA containing 20 mM GA, $4.84 \pm 0.01$ (n=2) for SA containing 0.5 mM $H_2O_2$, and $4.91 \pm 0.08$ (n=3) for SA containing 1 mM DI as shown in Figure 7 and Table 1. The degree of crosslinking at the pI values was not assessed.

3-d. Extractibility of Modified SA

At low ionic strength, membranes treated with 20 mM GA was highly resistant to the extraction of SA. The yield of SA from the modified ghosts was 80% lower than unmodified control. There was also a change in composition of the low-ionic strength SA extract. The new composition was 82.4% crosslinked and 8.1% non-crosslinked spectrin, and 9.5% actin.

3-e. Weakly immobilized motions in spin-labeled SA systems.

1. The W and S amplitudes of spin-labeled SA

The conventional EPR spectrum of Mal-6 labeled SA at pH 7.4 and 20 °C reveals overlapping weakly and strongly immobilized signals (Figure 8). In the spectrum, the narrow line component (W) is associated with weakly immobilized motions and the broad line component (S) is associated with strongly immobilized motions. Similar composite spectra were obtained for Mal-6 labeled SA when treated with 20 mM GA, 1 mM DI, 0.5 mM $H_2O_2$ and 20 mM PH.
Figure 7. The solubility behavior of modified spectrin-actin samples at low pH and 20 °C. After modification, the pH of the samples were adjusted by addition of 1N HCl and the absorbance were obtained at various pH points as described in the Materials and Methods.
Figure 8. The conventional EPR spectra of Mal-6 spin labeled spectrin-actin (control and modified) in 5 mM sodium phosphate buffer at pH 7.4: a) SA, b) SA-GA, c) SA-DI, d) SA-H$_2$O$_2$, and e) SA-PH. The spectra were recorded at 20 °C. The amplitude signals W and S are indicated in the EPR spectrum of control spectrin-actin.
2. **WIS of SA-GA.**

GA modification of isolated SA led to a 49.4% increase in the EPR spectral parameter WIS, or about 4% conversion of s to w states. The W/S increased from $2.41 \pm 0.03$ (n=11) to $3.45 \pm 0.07$ (n=11) as shown in Figure 9a. On the other hand, ghosts treated with various concentrations of GA showed a decreasing W/S trend. The W/S decreased from $3.19 \pm 0.07$ (n=10) to $2.74 \pm 0.04$ (n=10). The 14% decrease in W/S corresponds to less than 2% conversion of w to s states. Figure 9b shows the effect of GA on SA at two different ionic strengths: 0 and 150 mM NaCl in 5 mM phosphate buffer pH 7.4. The W/S of the paired modified and unmodified SA-GA samples decreased from $3.45 \pm 0.03$ (n=3) to $2.58 \pm 0.03$ (n=3) in the presence of salt. The 25% decrease translates to a 2% conversion of w to s (Table 2).

3. **Change in W/S ratio of modified SA and $f_b$.**

The change in W/S ratio was also plotted with crosslinker concentration (Figure 10). It is seen that crosslinking of SA with increasing concentrations of GA, DI, H$_2$O$_2$, and PH gradually increased the spin-labeled motion of the modified region. The change in W/S ratio is related to $f_b$, which is the fraction of spin labeled SA sites that have crosslinker associated with them. The relationship is described in the Materials and Methods.

4. **The W/S ratio and motional compositions of modified SA.**

Increased W/S ratios were observed for the modified SA samples (Figure 10). The unmodified SA samples in 5P7.4 at 20 °C exhibited an average W/S value of $2.40 \pm 0.03$ (n=18). The average W/S value for SA-GA samples with 20 mM GA concentration was $3.45 \pm 0.07$ (n=11), for SA-DI samples with 1 mM DI concentration was $3.90 \pm 0.02$ (n=4), for
Figure 9.  (a) The W/S ratio of glutaraldehyde-treated Mal-6 labeled spectrin-actin and membrane ghosts at pH 7.4 and 20 °C. SA = spectrin-actin; Mb = membrane ghosts. (b) The W/S ratio of glutaraldehyde-treated Mal-6 labeled spectrin-actin at pH 7.4 and 20 °C at different ionic strengths. PBS = 150 mM NaCl in 5 mM phosphate buffer pH 7.4; 5P7.4 = 5 mM phosphate buffer pH 7.4.
Figure 10. The typical relationship at 20 °C between the concentration of the crosslinker and the change in W/S ratio of spectrin-actin upon treatment with a) glutaraldehyde, b) diamide, c) hydrogen peroxide, and d) phenyl hydrazine.
Figure 11. The conventional $\langle V_1 \rangle$ EPR spectrum at the isoelectric pH of Mal-6 labeled spectrin-actin modified with (a) 20 mM glutaraldehyde, (b) 1 mM diamide, (c) 0.5 mM hydrogen peroxide, and (d) 20 mM phenyl hydrazine. The spectra were recorded at 20 °C.
H$_2$O$_2$-SA samples with 0.5 mM H$_2$O$_2$ concentration was 2.65 ± 0.02 (n=4), and for SA-PH samples with 20 mM PH concentration was 3.74 ± 0.05 (n=3). Thus the SA-DI samples exhibit the largest increase in W/S values and the SA-H$_2$O$_2$ samples exhibit the lowest increase in W/S values.

Since the w signal is a narrow signal whereas the s signal is a broad signal, a large increase in W/S ratio may represent only a small percentage of conversion of s to w [48]. We also determined the composition of the w and s in each paired unmodified and modified samples. The unmodified SA samples with the W/S ratio of 2.39 ± 0.03 (n=3) consists of 89.6 ± 0.4 % (n=3) and 10.4 ± 0.4 % (n=3). This motional composition (%s and %w) was determined by both spectral subtraction, and use of slope and intercept of the literature calibration curve [46]. In the paired data of the modified samples, SA-GA consists of 86.3 ± 0.4 % s (n=11) and 13.7 ± 0.4 % w (n=11) due to a change of 43.8 ± 6.7 % (n=3) in W/S. SA-DI consists of 84.8 ± 0.2 % s (n=4) and 15.2 ± 0.2 % w (n=4) resulting from a 62.5 ± 2.7 % (n=3) change in W/S. SA-H$_2$O$_2$ consists of 89.1 ± 0.2 % s (n=4) and 10.9 ± 0.2 % w (n=4) upon a 10.4 ± 3.7 % (n=4) change in W/S. SA-PH consists of 85.3 ± 0.5 % s (n=3) and 14.7 ± 0.5 % w (n=3) after a 56.5 ± 3.5 % (n=3) change in W/S. It was seen that the fast component, w, of the oxidized systems ranged from 11-15 % of the total signal intensity.

A summary of % w and % s is given in Table 2.

5. **Conventional EPR spectrum of modified SA at low pH**

Relatively broad spectral lines and powder pattern spectra were exhibited by SA-GA, SA-DI, SA-H$_2$O$_2$, and SA-PH at 20 °C at their pI. There was little or no significant difference in the linewidth and the hyperfine separations of the extremal lines of the modified samples (Figure 11).
TABLE 2.

Summary of the motional compositions of modified spectrin-actin systems and control.

Percent w and s signals in the conventional EPR spectra were obtained from the W/S ratios using the method of Fung [142].

<table>
<thead>
<tr>
<th>System</th>
<th>% w</th>
<th>% s</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-Buffer</td>
<td>10.4 ± 0.4 (n=11)</td>
<td>89.6 ± 0.4 (n=11)</td>
</tr>
<tr>
<td>SA-GA (20 mM)</td>
<td>13.7 ± 0.4 (n=11)</td>
<td>86.3 ± 0.4 (n=11)</td>
</tr>
<tr>
<td>SA-DI (1 mM)</td>
<td>15.2 ± 0.2 (n=4)</td>
<td>84.8 ± 0.2 (n=4)</td>
</tr>
<tr>
<td>SA-H₂O₂ (0.5 mM)</td>
<td>10.9 ± 0.2 (n=4)</td>
<td>89.1 ± 0.2 (n=4)</td>
</tr>
<tr>
<td>SA-PH (20 mM)</td>
<td>14.7 ± 0.5 (n=3)</td>
<td>85.3 ± 0.5 (n=3)</td>
</tr>
</tbody>
</table>

W/S = amplitude ratio;  w = concentration of spin labels associated weakly immobilized motions;  s = concentration of spin labels that are associated with strongly immobilized motions.
6. **Conventional EPR spectra and spectral subtraction**

Representative conventional EPR spectra of modified SA samples and controls at 4 °C and pH 6 are shown in Figure 12. The spectra shown were all normalized to the same signal amplitude, and therefore do not reflect differences in the individual spin label intensities.

Using 1 mM DI-modified, spin-labeled SA and control at 20 °C and pH 8 and pH 6 (top row), spectral subtraction removes the fast motional component (center) to give slow motional components that have only small differences in spectral features.

3-f. **Strongly immobilized motions in spin labeled SA systems**

Representative second harmonic, 90° out of phase, absorption X-band ST-EPR spectra of SA, SA-GA and SA-DI are shown in Figure 13. Each of these composite spectra are separated into two components: the fast and slow motional components (Figure 14). In Figure 10, 1 mM DI-modified, Mal-6 labeled SA recorded at 20 °C at pH 8 and pH 6 are given (top row). The spectra shown are all normalized to the same line height and therefore do not reflect the differences in relative intensities. After spectral subtraction [74] to remove the fast motional component, the resulting slow motion spectra of SA-DI at pH 6 and pH 8 were qualitatively similar.

3-g. **Signal amplitude ratios and rotational correlation times**

To treat the $V'_2$ spectra of SA, SA-GA, SA-DI, SA-$H_2O_2$ and SA-PH quantitatively, the line height ratio parameters employed by Thomas et. al. [89] were used (Figure 15). The signal amplitude ratios, $L''/L$, $C'/C$, and $H''/H$ exhibit dependence on rotational motions of proteins. In each case, the numerator is the signal amplitude that is most sensitive to motion within a spectral region, and the denominator is the least sensitive [152, 89].
Figure 12. Spectral subtraction using the conventional EPR spectra of Mal-6 labeled, 1 mM DI-treated spectrin-actin (concentration about 12 mg/ml) at 20 °C. The top row spectra which were recorded at pH 8 and pH 6, respectively contain different proportions of fast and slow motional components. By spectral subtraction (Fung & Johnson, 1983), the fast motional component (center) and the slow motional component (bottom) spectra were obtained.
Figure 13. The X-band ST-EPR composite spectra of Mal-6 labeled modified spectrin-actin and spectrin-actin control: a) SA, b) SA-GA, and c) SA-DI. The spectra were recorded at pH 6 and 20 °C.
Figure 14. Spectral subtraction using the X-band ST EPR spectra of Mal-6 labeled, 1 mM DI-modified spectrin-actin (about 12 mg/ml) at 20 °C. The top row spectra which were recorded at pH 8 and pH 6, respectively, contained different proportions of fast and slow motional components. Using spectral subtraction by Fung & Johnson (1983), the fast motional component (center) and the slow motional component (bottom) spectra were obtained.
pH 8

pH 6

Weakly immobilized component
Figure 15. The slow motion X-band ST EPR spectrum of 1 mM DI-treated Mal-6 labeled spectrin-actin at pH 6 and 20 °C obtained after spectral subtraction. The motionally sensitive ratio parameters, $L''/L$, $C'/C$, and $H''/H$ were measured from the positions indicated using the procedures of Thomas et. al. (1976).
Table 3 compares the diagnostic signal amplitude ratios and rotational correlation times of SA, SA-GA, and SA-DI at pH 6 and 20 °C obtained after spectral subtraction (Figure 16).

The L"/L ratios of 0.74 ± 0.02 (n = 3) for SA, 0.58 ± 0.01 (n = 3) for SA-GA, 0.65 ± 0.12 (n = 3) for SA-DI correspond to rotational correlation times, $\tau_L$, of 7.8 ± 0.7 x $10^{-5}$ sec (n = 3), 2.8 ± 0.2 x $10^{-5}$ sec (n = 3), and 5.0 ± 1.9 x $10^{-5}$ sec (n = 3), respectively. The C'/C ratios of -0.91 ± 0.02 (n = 3) for SA and -0.95 ± 0 (n = 3) for SA-DI were slightly higher than -0.75 ± 0.04 (n = 3) for SA-GA. In this region, the rotational correlation times, $\tau_c$, of 1.2 ± 0.2 x $10^{-7}$ (n = 3) sec for SA and 1.1 ± 0.4 x $10^{-7}$ sec (n = 3) for SA-DI were slightly lower than 2.5 ± 0.3 x $10^{-7}$ sec (n = 3) for SA-GA. The H"/H ratios of 0.37 ± 0 (n = 3) for SA and 0.34 ± 0.02 (n = 3) for SA-GA were lower than that of SA-DI (H"/H of 0.42 ± 0.03, n = 3). The rotational correlation times, $\tau_H$, of 4.6 ± 0.2 x $10^{-5}$ sec (n = 3) for SA and 4.2 ± 0.3 x $10^{-5}$ sec (n = 3) for GA-SA were very slightly lower than that of SA-DI (6.4 ± 1.0 x $10^{-5}$ sec, n = 3). The correlation times of SA and SA modified with GA or DI are consistent with superimposition of motions.

The signal amplitude ratios of SA, SA-GA and SA-DI under different conditions before spectral subtraction were also obtained (Table 4). At pH 6 and 20 °C (Table 4a, Figure 13), the L"/L ratio 0.76 ± 0.01 (n = 3) for SA was slightly higher than SA-GA (0.66 ± 0.01, n = 3) but lower than SA-DI (0.90 ± 0.04, n = 3). On the other hand, the C'/C ratios of -1.13 ± 0.01 (n = 3) for SA and -1.15 ± 0.14 (n = 3) for SA-DI were similar, but were slightly lower than -0.90 ± 0.14 (n = 3) for SA-GA. The H"/H ratios of 0.31 ± 0.01 (n = 3) for SA were lower than that of SA-GA (0.46 ± 0.01, n=3) and SA-DI (0.47 ± 0.02, n=3). At pH 8 and 20 °C (Table 4, Figure 17), the L"/L ratio 0.94 ± 0.03 (n = 5) for SA was lower than that of SA-GA (1.52 ± 0.04, n = 5) and SA-DI (1.73 ± 0.06, n = 3). On the other hand, the C'/C ratios of -1.93 ± 0.12 (n = 5) for SA, -1.83 ± 0.20 (n = 5) for SA-GA, and -1.75 ± 0.19
TABLE 3.

**Summary of the signal amplitude ratios of modified spectrin-actin systems at pH 6 and 20°C.**

The signal amplitude ratios $L''/L$, $C'/C$, and $H''/H$ and rotational correlation times were obtained from the ST-EPR spectra of the weakly immobilized components removed after spectral subtraction according to the published methods [71, 89]. The values are the averages of three experiments.

<table>
<thead>
<tr>
<th>System</th>
<th>$L''/L$</th>
<th>$\tau_L \times 10^5$ (sec)</th>
<th>$C'/C$</th>
<th>$\tau_C \times 10^5$ (sec)</th>
<th>$H''/H$</th>
<th>$\tau_H \times 10^5$ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA slow</td>
<td>0.74 ± 0.02</td>
<td>7.8 ± 0.7</td>
<td>-0.91 ± 0.02</td>
<td>1.2 ± 0.2</td>
<td>0.37 ± 0.00</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>SA-GA slow</td>
<td>0.58 ± 0.01</td>
<td>2.8 ± 0.3</td>
<td>-0.75 ± 0.04</td>
<td>2.5 ± 0.3</td>
<td>0.34 ± 0.02</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>SA-DI slow</td>
<td>0.65 ± 0.12</td>
<td>5.0 ± 1.9</td>
<td>-0.95 ± 0.00</td>
<td>1.1 ± 0.4</td>
<td>0.42 ± 0.03</td>
<td>6.4 ± 1.0</td>
</tr>
</tbody>
</table>

The samples consisted of 1 mg/ml spectrin-actin (SA) with or without the following chemicals: 20 mM glutaraldehyde (GA), or 1 mM diamide (DI) in 5 mM phosphate buffer at pH 6 and 20°C.

$L''/L$, $C'/C$, and $H''/H$ are the signal amplitude ratios in the low-field, center, and high field regions of the slow motion ST-EPR spectrum, respectively.

$\tau_L$, $\tau_C$, and $\tau_H$ are correlation times at the low, center, and high field regions, respectively.
Figure 17. The X-band ST-EPR composite spectra of Mal-6 labeled spectrin-actin control.

The spectra was recorded at pH 8 and 20 °C: a) SA, b) SA-GA, and c) SA-DI.
TABLE 4.

Summary of the signal amplitude ratios of modified spectrin-actin systems and control at (a) pH 6 and 20 °C; (b) pH 8 and 20 °C.

The signal amplitude ratios \( L'/L \), \( C'/C \), and \( H'/H \) and rotational correlation times were obtained according to the published methods [71, 89] from the composite ST-EPR spectra before spectral subtraction. The values presented are the averages of at least three experiments.

<table>
<thead>
<tr>
<th>System</th>
<th>( L'/L )</th>
<th>( C'/C )</th>
<th>( H'/H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>0.76 ± 0.01</td>
<td>-1.13 ± 0.01</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>SA-GA</td>
<td>0.66 ± 0.01</td>
<td>-0.90 ± 0.05</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>DI-SA</td>
<td>0.90 ± 0.04</td>
<td>-1.15 ± 0.14</td>
<td>0.47 ± 0.02</td>
</tr>
</tbody>
</table>

a)

<table>
<thead>
<tr>
<th>System</th>
<th>( L'/L )</th>
<th>( C'/C )</th>
<th>( H'/H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>0.94 ± 0.03 (n=5)</td>
<td>-1.93 ± 0.12 (n=5)</td>
<td>0.41 ± 0.03 (n=5)</td>
</tr>
<tr>
<td>SA-GA</td>
<td>1.52 ± 0.04 (n=5)</td>
<td>-1.83 ± 0.20 (n=5)</td>
<td>0.61 ± 0.04 (n=5)</td>
</tr>
<tr>
<td>SA-DI</td>
<td>1.73 ± 0.06 (n=3)</td>
<td>-1.75 ± 0.19 (n=3)</td>
<td>0.48 ± 0.04 (n=3)</td>
</tr>
</tbody>
</table>

The samples consisted of 1 mg/ml spectrin-actin (SA) with or without the following chemicals: 20 mM glutaraldehyde (GA), or 1 mM diamide (DI) in 5 mM phosphate buffer at 20 °C and at the pH indicated in the table.

In both tables, \( L'/L \), \( C'/C \), and \( H'/H \) are the signal amplitude ratios in the low field, center field, and high field regions of the spectrum, respectively.
(n = 3) for SA-DI were similar. The $H''/H$ ratios of $0.41 \pm 0.03$ (n=5) for SA and $0.48 \pm 0.04$ (n = 3) for SA-DI were slightly lower than that of SA-GA ($0.61 \pm 0.04$, n=5). It was seen that the values of the $L''/L$, $C'/C$, and $H''/H$ at pH 6, 20 °C for SA, SA-GA, SA-DI, SA-H$_2$O$_2$ and SA-PH were lower than the corresponding ratios at pH 8, 20 °C. These values were consistent with superimposed motions.

Table 5 shows the diagnostic signal amplitude ratios and the slightly longer rotational correlation times of SA compared to SA-GA and SA-DI at pH 8 and 20 °C obtained after spectral subtraction (Figure 18). The $L''/L$ ratios of $0.72 \pm 0$ (n = 3) for SA, $0.57 \pm 0.01$ (n = 3) for SA-GA, and $0.63 \pm 0.12$ (n = 3) for DI-SA gave rotational correlation times, $\tau_L$, of $6.8 \pm 0.3 \times 10^{-5}$ sec (n = 3), $2.8 \pm 0.3 \times 10^{-5}$ sec (n = 3), and $5.0 \pm 2.1 \times 10^{-5}$ sec (n = 3), respectively. The $C'/C$ ratios of $-0.96 \pm 0.07$ (n =3) for SA, $-0.79 \pm 0.03$ (n = 3) for SA-GA, $-0.93 \pm 0.09$ (n =3) for SA-DI were not significantly different. In the center field region, the rotational correlation times, $\tau_C$, were $1.5 \pm 0.2 \times 10^{-7}$ (n = 3) sec for SA and $1.6 \pm 0.4 \times 10^{-7}$ sec (n = 3) for SA-DI, which were just slightly lower than that of SA-GA whose rotational correlation time was $2.4 \pm 0.2 \times 10^{-7}$ sec (n = 3). The $H''/H$ ratios of $0.37 \pm 0$ (n = 3) for SA and $0.35 \pm 0.01$ (n = 3) for SA-GA were slightly lower than that of SA-DI ($H''/H$ of $0.45 \pm 0.02$, n = 3). The rotational correlation times, $\tau_L$, of $4.8 \pm 0.3 \times 10^{-5}$ sec (n = 3) for SA and $4.6 \pm 0.1 \times 10^{-7}$ sec (n = 3) for SA-GA were lower than the rotational correlation time of SA-DI ($7.5 \pm 0.8 \times 10^{-7}$ sec, n = 3). The rotational correlation times of the modified SA and control were again consistent with superimposition of motions.

The signal amplitude ratios and correlation times of SA, SA-GA, SA-DI, SA-H$_2$O$_2$ and SA-PH before spectral subtraction at pH 6 and 4 °C are compared in Table 6 (Figure 19). The $L''/L$ ratios of $0.65 \pm 0.04$ (n = 3) for SA, $0.62 \pm 0.01$ (n = 3) for GA-SA, $0.74 \pm 0.03$ (n= 3) for DI-SA, $0.64 \pm 0.03$ (n = 3) and $0.68 \pm 0.06$ (n = 3) for PH-SA were similar.
The C'/C ratios of -0.76 ± 0.10 (n = 3) for SA, -0.82 ± 0.02 (n = 3) for GA-SA, -0.76 ± 0.03 (n = 3) for DI-SA, -1.01 ± 0.10 (n = 3) for H$_2$O$_2$-SA, and -0.91 ± 0.11 (n = 3) were not significantly different. The H'/H ratio of 0.67 ± 0.03 (n = 3) for SA was higher than that of GA-SA, DI-SA, H$_2$O$_2$-SA and PH-SA whose values were 0.36 ± 0.01 (n = 3), 0.44 ± 0.01 (n = 3), 0.30 ± 0.02 (n = 3), and 0.43 ± 0.06 (n = 3), respectively. The values of rotational correlation times obtained from the unsubtracted spectra at pH 6 and 4 °C were similar to those obtained from the unsubtracted spectra at pH 6-8 and 20 °C. The rotational correlation times of the modified systems were consistent with superimposition of motions.
Figure 18. The slow motion X-band ST-EPR spectra of Mal-6 labeled spectrin-actin and modified spectrin-actin at pH 8 and 20 °C after spectral subtraction: a) SA, b) SA-GA, and c) SA-DI.
Summary of the signal amplitude ratios of modified spectrin-actin systems at pH 8 and 20°C.

The signal amplitude ratios $L''/L$, $C'/C$, and $H''/H$ and rotational correlation times were obtained from the ST-EPR spectra of the weakly immobilized components removed after spectral subtraction according to the published methods [71, 89]. The values are the averages of three experiments.

<table>
<thead>
<tr>
<th>System</th>
<th>$L''/L$</th>
<th>$\tau_L \times 10^5$ (sec)</th>
<th>$C'/C$</th>
<th>$\tau_C \times 10^5$ (sec)</th>
<th>$H''/H$</th>
<th>$\tau_H \times 10^5$ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA slow</td>
<td>0.72 ± 0.00</td>
<td>6.8 ± 0.3</td>
<td>-0.96 ± 0.07</td>
<td>1.5 ± 0.2</td>
<td>0.37 ± 0.01</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>SA-GA slow</td>
<td>0.57 ± 0.01</td>
<td>2.8 ± 0.3</td>
<td>-0.79 ± 0.03</td>
<td>2.4 ± 0.2</td>
<td>0.35 ± 0.01</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>SA-DI slow</td>
<td>0.63 ± 0.12</td>
<td>5.0 ± 2.1</td>
<td>-0.93 ± 0.09</td>
<td>1.6 ± 0.4</td>
<td>0.45 ± 0.02</td>
<td>7.5 ± 0.8</td>
</tr>
</tbody>
</table>

The samples consisted of 1 mg/ml spectrin-actin (SA) with or without the following chemicals: 20 mM glutaraldehyde (GA), or 1 mM diamide (DI) in 5 mM phosphate buffer at pH 8 and 20 °C.

$L''/L$, $C'/C$, and $H''/H$ are the signal amplitude ratios in the low-field, center, and high field regions of the slow motion ST-EPR spectrum, respectively.

$\tau_L$, $\tau_C$, and $\tau_H$ are correlation times at the low, center, and high field regions, respectively.
Figure 19. The X-band ST-EPR composite spectra of Mal-6 labeled spectrin-actin control. The spectra was recorded at pH 6 and 4 °C. a) SA, b) SA-GA, c) SA-DI, d) SA-H₂O₂, and e) SA-PH.
TABLE 6.

Summary of the signal amplitude ratios of modified spectrin-actin systems at pH 6 and 4 °C.

The signal amplitude ratios L"/L, C'/C, and H"/H and rotational correlation times shown with square brackets ([ ]) were obtained using the published methods from the ST-EPR spectra exhibiting mostly strongly immobilized components, and thus spectral subtractions were not performed. The values are the averages of three experiments.

<table>
<thead>
<tr>
<th>System</th>
<th>L&quot;/L</th>
<th>τ_L x 10^5 (sec)</th>
<th>C'/C</th>
<th>τ_C x 10^5 (sec)</th>
<th>H&quot;/H</th>
<th>τ_H x 10^5 (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>0.65 ± 0.04</td>
<td>[5.2 ± 1.3]</td>
<td>-0.76 ± 0.10</td>
<td>[2.6 ± 1.5]</td>
<td>0.39 ± 0.03</td>
<td>[5.5 ± 0.7]</td>
</tr>
<tr>
<td>SA-GA</td>
<td>0.62 ± 0.01</td>
<td>[4.3 ± 0.2]</td>
<td>-0.72 ± 0.03</td>
<td>[2.7 ± 1.0]</td>
<td>0.44 ± 0.01</td>
<td>[7.3 ± 0.3]</td>
</tr>
<tr>
<td>SA-DI</td>
<td>0.74 ± 0.03</td>
<td>[7.7 ± 0.8]</td>
<td>-0.82 ± 0.02</td>
<td>[2.1 ± 0.5]</td>
<td>0.36 ± 0.01</td>
<td>[4.5 ± 0.9]</td>
</tr>
<tr>
<td>SA-H₂O₂</td>
<td>0.64 ± 0.03</td>
<td>[4.6 ± 0.6]</td>
<td>-1.01 ± 0.10</td>
<td>[1.4 ± 0.2]</td>
<td>0.30 ± 0.02</td>
<td>[2.9 ± 0.9]</td>
</tr>
<tr>
<td>SA-PH</td>
<td>0.68 ± 0.06</td>
<td>[5.9 ± 1.7]</td>
<td>-0.91 ± 0.11</td>
<td>[1.7 ± 0.6]</td>
<td>0.43 ± 0.06</td>
<td>[7.3 ± 2.2]</td>
</tr>
</tbody>
</table>

The samples consisted of 1 mg/ml spectrin-actin (SA) with or without the following chemicals: 20 mM glutaraldehyde (GA), 1 mM diamide (DI), 0.5 mM H₂O₂, or 20 mM phenylhydrazine (PH) in 5 mM phosphate buffer at pH 8 and 20 °C.

L"/L, C'/C, and H"/H are the signal amplitude ratios in the low-field, center, and high field regions of the slow motion ST-EPR spectrum, respectively.

τ_L, τ_C, and τ_H are correlation times at the low, center, and high field regions, respectively.
CHAPTER 4

DISCUSSION

Proteins are dynamic, flexible molecules. Thermally driven motions influence every aspect of their structure. Bonds vibrate, chains rotate, surface loops and segments wiggle, protein domains flex about their connecting links, and the entire structure breathes by opening and closing about the most stable conformation [96]. These internal motions provide specific dynamic foundations for a lot of protein functions [97, 98]. These motions occur on time scales of picoseconds (bond vibrations), nanoseconds (segmental motions and isotropic rotation of small proteins), microseconds (segmental domain motion of large proteins), milliseconds (rotation of large asymmetric proteins and flexing of filamentous proteins) and seconds (protein folding) [99].

SA, the major determinant of erythrocyte membrane shape and deformability, is highly flexible and elastic [41, 100-102]. It exhibits multiple classes or rates of motion which are classified as fast, slow and very slow motions. The three motional components appear to have correlation times, \( \tau \leq 10^{-9} \) sec, \( 10^{-7} - 10^{-6} \) sec, and about \( 10^{-3} \) sec [74]. The presence of fluctuational segmental motions in SA have been confirmed by other physical methods [74-81]. The segmental motions detected by spin label EPR methods appear to be the same for SA in solution or in membranes [74-76], indicating that attachment of SA to other proteins and/or lipid bilayer at their anchoring postions did not alter the segmental motions of SA. However, substantial immobilization of SA was observed in samples at low pH [103-105].
Spectrin can be crosslinked [52, 54, 62, 67, 87]. Spectrin crosslink formation appears to be specific, demonstrates a dose response, and represents a biochemical event that may be related to the molecular/pathogenic events in the RBC [67]. But how the degree of crosslinking affects the molecular dynamics and conformational flexibility of SA is not known. Since it is often assumed that spectrin flexibility is the molecular origin of the unique deformability and elasticity of the human erythrocyte, we investigated the segmental motions of SA crosslinked with GA, DI, H$_2$O$_2$ and PH using conventional and ST-EPR and employing Mal-6, an NEM analog.

4-a. **The degree of crosslinking in SA by SDS-PAGE**

GA was used because it is a very common reagent to preserve biological specimen. It reacts primarily with the $\epsilon$-NH$_2$ group of lysine to give a crosslink that is stable to acid hydrolysis (Figure 20). The stability towards hydrolysis seem to be provided by the interaction of the Schiff base with the adjacent double bonds [106]. GA (0.5 %) can partially react with SH groups [107, 108], tyrosine and histidine of proteins [108]. Pathways involving the reactions of the amino groups with $\alpha$, $\beta$-unsaturated aldehydes formed by the aldol condensations of GA have been proposed [109, 110]. Others have postulated pathways involving the formation of quaternary pyridinium compounds [111, 112]. Therefore, the exact mechanism of the GA reaction is not very well established. GA has very mild physical effects on membrane proteins [113].

At 22 °C and pH 8, 1-3 mM GA crosslinks spectrin in exclusively in erythrocyte membranes to form HMW complexes in the 400 and 500 kD region, and it did not affect sulphydryl groups [62]. The extent of crosslinking was not given in this study. We have carried out similar studies with GA in the 1-3 mM range in isolated SA, rather than membranes, and obtained very similar results based on the dose dependent nature of GA reaction with SA and
the molecular weights of the complexes formed. We have also extended the studies to determine the saturating levels of GA to give maximum crosslinking. At 20 mM GA, about 80% of the spin labeled SA were crosslinked to form high molecular weight complexes. The HMW species obtained by treating SA with at saturating level represented multiple spectrin heterodimers based on molecular weight estimates. This formation of multiple species of spectrin is consistent with the existence of several sites in spectrin that are more preferentially crosslinked than other sites [67]. The concentrations of GA used in our studies ranged from 1 - 20 mM which are much lower than those used to fix tissues and proteins (100 - 300 mM), and slightly higher than Steck's (1 - 3 mM). In this study, the degree of SA crosslinking did not change at the duration of the experiment based on SDS-PAGE indicating irreversibility of the crosslinks formed. This irreversibility is consistent with the published [106, 107, 111, 115]. The nature of the glutaraldehyde reaction with proteins is shown in Figure 20.
Figure 20. The structure and reaction of glutaraldehyde with (a) predominant form at acid pH, (b) predominant form at neutral pH, and (c) conjugated Schiff base product between glutaraldehyde and protein amino acids (Monsan et. al., 1975; Peters and Richards, 1977).
a) $\text{CHO} \rightarrow \text{CHO}$

b) $\text{CHO} \rightarrow \left[ \text{CHO} \right] \rightarrow \left[ \text{CHO} \right] \rightarrow \left[ \text{CHO} \right]$

d) $\text{CHO} + \text{Protein} \rightarrow \text{NH}_2$

e) $\text{CHO} + \text{Protein} \rightarrow \text{NH}_2$

f) $\text{CHO} + \text{Protein} \rightarrow \text{NH}_2$
Diamide (DI), a bifunctional SH reagent, has been shown to stoichiometrically oxidize the sulphydryl group of cysteine in red cell glutathione and in proteins to the disulfide without generating radicals in aqueous solutions at neutral pH [115, 55]. It has been shown to inhibit echinocyte-discocyte transformation [116]. Varying degrees of disulfide bond formation resulting from spectrin oxidation have been observed at 37 °C and pH 7.4. At low concentrations (about 50 µM), with only 5% membrane SH modified, a 50% decrease in elongation of erythrocytes when subjected to viscometric flow was detected [117]. For erythrocytes treated with 5 mM DI, with only 5% of β spectrin crosslinked, increase in membrane stiffness was observed [118]. In intact RBC, diamide exclusively crosslinks spectrin via disulfide bonds. The glutathione in erythrocytes protects band 3, but not spectrin, from forming disulfide bonds [52]. For isolated spectrin treated with 2.5 µM DI, the binding of spectrin to band 4.1 was altered [3]. At this concentration, no other functional alterations were detected. Circular dimers or circular single chains were detected at low concentrations, and mixture of rings and extended structures were detected at higher DI concentrations (2 - 5 mM) [3]. However, no difference in tryptic digestion pattern was observed in DI-treated spectrin samples. Becker and co-workers [3] suggested that oxidation leading to disulfide bond formation might produce secondary local conformational changes in proteins. However in our studies, extensive crosslinking by DI (over 60% SA was crosslinked at 1 mM saturating concentration of DI) caused the pl value to change by about 0.4 pH units (Section 3-c), but did not cause any major alteration in segmental motions in SA as detected by ST EPR (Section 3-g). The extent of spectrin crosslinking in our studies 1 mM DI was within the range of those reported. In those studies different buffer compositions [3, 54, 118] and pH [117] were used. The extent of SA crosslinking was concentration-dependent (Figure 1b). The crosslinks were probably spectrin β-chain dimer, α-chain dimer, and mixed α-β heterodimer based on molecular weight estimates.
Bennett et al. [119] reported that the formation of mixed $\alpha$-$\beta$ heterodimers is highly favored over $\alpha$-$\alpha$ or $\beta$-$\beta$ associations. In our studies, it is therefore, possible that spectrin chains ($\alpha$ and $\beta$) became separated during oxidant modification, and then aggregated to contain pure chain, pure $\beta$-chain, or $\alpha$-$\beta$ chains. In fact, it has been reported that isolated $\beta$-chains aggregate [89], although this has not been generally confirmed by others [119-121]. Since our crosslinking was done in the absence of DTT, the HMW species may be linked by intermolecular disulfide bonds.

We have also compared the degree of crosslinking in SA at different temperatures to determine the optimum condition for DI-induced oxidation.

Crosslinking at 37°C was 20% higher than at 20°C at pH 7.4 (not shown). The crosslinks obtained at the two temperatures were identical based on their relative mobilities. We have, therefore used 37°C to modify the SA samples. Becker et al. [3] showed that at 0°C and pH 8, micromolar amount of DI caused 10% of spectrin to form HMW species. Our results at 0°C and pH 7.4 using 1 mM DI showed that 30% of the spectrin was oxidized. Our results supplement the published [3] even if the concentrations of DI, the kind of buffer used and the pH we used were different. The irreversible reaction of DI with sulfhydryl groups of proteins is shown in Figure 21.
Figure 21. The preferential reaction of diamide with protein sulphydryl groups at pH 7.4 to form disulfide bonds (Kosower et. al., 1969).
$\text{(CH}_3\text{)}_2\text{NC} = \text{N} = \text{N} - \text{CN}(\text{CH}_3)_2 + 2\text{RSH} \rightarrow$

RSSR + $\text{(CH}_3\text{)}_2\text{NC} - \text{NH} - \text{NH} - \text{CN}(\text{CH}_3)_2$
When erythrocytes were treated with very high levels (147 mM) of \( \text{H}_2\text{O}_2 \), no change in cell volume distribution, filterability and membrane protein electrophoresis pattern occurred [11]. Most of the published studies on \( \text{H}_2\text{O}_2 \) with spectrin were focused on the formation of spectrin-hemoglobin aggregates [59, 64, 122]. We have used 0.5 mM \( \text{H}_2\text{O}_2 \) to oxidize isolated SA and found 60% of SA formed high molecular weight aggregates. Our results are consistent with [123] but differed from those of Sauberman et. al. [64]. It must be stressed that the oxidizing conditions that we used were different. Thiols are converted to disulfides by \( \text{H}_2\text{O}_2 \) but oxidation could go beyond the disulfide if the thiol groups are sterically free to interact with one another [119] and if higher concentrations and low pH are used [118]. Based on SDS-PAGE molecular weight estimates, and under the experimental conditions used (no DTT), the SA-\( \text{H}_2\text{O}_2 \) species that we obtained were probably disulfide crosslinks. The mechanism of action of \( \text{H}_2\text{O}_2 \) with SH groups is shown in Figure 22.

The capacity of phenyl hydrazine to form \( \text{H}_2\text{O}_2 \) has been known [124]. Reactive intermediates can be formed from the autooxidation of PH and \( \text{H}_2\text{O}_2 \) is a major product [125, 126]. Phenylhydrazine treatment of red blood cells causes the breakdown of hydroperoxides from lipid peroxidations to produce malondialdehyde which promotes protein crosslinking [6] and increased lipid fluidity [4]. High molecular weight complexes derived from spectrin were extracted in erythrocytes treated with PH [127]. Phenylhydrazine also produced activated oxygen species [125] to degrade spectrin [65, 66, 129]. Decreased spectrin and formation of HMW species were also observed in membranes modified with 5 mM PH [65] but spectrin does not form HMW disulfides at a PH concentration of 1 mM or lower [66]. Our results (Figure 1d) shows that the polymerization of SA is dose-dependent, and at saturating level of PH (20 mM), about 60% of SA formed HMW complexes at 37 °C and pH 7.4. This is consistent with the published [65, 129]. Figure 23 shows the formation of active species and \( \text{H}_2\text{O}_2 \) from PH.
Figure 22. The mechanism of action of hydrogen peroxide. The formation of the product depends on the conditions used (O'Brien and Little, 1969).
\[
\begin{align*}
[O^-] + RSH & \xrightarrow{k_1} RS^- + OH^- \\
RS^- + RS^- & \xrightarrow{k_2} RSSR \\
RS^- + 6[O^-] + 6H^+ & \xrightarrow{k_3} R-S-OH + 3H_2O \\
[O^-] &= 1 \text{ equivalent of } H_2O_2
\end{align*}
\]
Figure 23. The formation of reactive intermediates from the autooxidation of phenyl hydrazine and the generation of $\text{H}_2\text{O}_2$ (Misra and Fridovich, 1976).
(a) Autoxidation in aqueous solution:

\[
\text{NENH}_2^+ + \text{O}_2 \rightarrow \text{NENH}^+ + \text{H}^+ + \text{N}=\text{NH}^-
\]

(b) Reaction with oxygen:

\[
\text{NENH}^+ + \text{O}_2 \rightarrow \text{H}^+ + \text{O}_2^- + \text{NENH}_2
\]

(c) Formation of hydrogen peroxide:

\[
\text{O}_2^- + \text{H}^+ + \text{NENH}_2 \rightarrow \text{H}_2\text{O}_2 + \text{NENH}^+ + \text{NENH}_2
\]

(d) Reaction of hydrogen peroxide with radical:

\[
2 \text{NENH}^+ \rightarrow \text{NENH}_2 + \text{N}=\text{NH}^-
\]

(e) Formation of superoxide:

\[
\text{N}=\text{NH}^- + \text{O}_2 \rightarrow \text{N}=\text{N}^- + \text{H}_2\text{O}_2
\]

(f) Decomposition of nitrogen:

\[
\text{N}=\text{N}^- \rightarrow \text{N}^+ + \text{N}_2
\]

(g) Reaction with substrate:

\[
\text{N}^+ + \text{substrate-H} \rightarrow \text{N}^- + \text{substrate}
\]

(h) Formation of hydrogen peroxide:

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2
\]
4-b. Differences in optical absorption of modified SA

Changes in light absorption can be brought about by the formation of protein polymers. These changes may occur in two cases: 1) when chromophore groups (e.g., tryptophan, tyrosine and phenyl alanine) are in the region of intermolecular contact and in the course of complex formation, they are transferred from the aqueous environment to the interior of lower polarity or into conditions of other specific interactions with the environment of protein groups; 2) when the formation of associates may alter the conformation of the subunits which leads to changes in the environment of chromophores groups which are not always at the surface of the protein molecule. In both cases, a shift in the absorption spectrum can be expected [130]. Changes in light absorption may also be induced by changes in solvent properties even in the absence of conformational changes, and by the interaction of proteins with small molecules and ions. The exposed chromophore groups of proteins respond to the changes in solvent properties which perturb their absorption spectra [130, 131]. These have been demonstrated in a lot of UV studies on the polymerization of various proteins like insulin, glucagon, hemoglobin, lysozyme and G-actin [132].

Having confirmed the presence of spectrin polymers in the SA treated with GA, DI, H₂O₂ or PH, we investigated the optical absorption of properties the modified SA molecules. Pure SA absorbs at 280 nm. The change UV absorption spectra at pH 7.4 showing λₘₐₓ shifts of 8 nm, 1 nm, 3 nm and 2nm for SA-GA, SA-DI, SA-H₂O₂ and SA-PH, respectively, which were accompanied by changes in extinction coefficient and increased absorbance. These properties may be due to one or more reasons mentioned above. Habeeb & Hiramoto [108] has shown that while GA reacts primarily with lysine, it can also react with some tyrosines. Salmine, devoid of tyrosine and tryptophan, does not exhibit such UV spectral change when treated with GA. In the same study, sterically hindered tyrosines were suggested for GA-ovalbumin, a result that
was in support of a previous study by Sokolovesly & Vallee [132]. The change in UV spectra of SA modified with 20 mM GA suggests changes in the environment of the chromophoric groups, particularly the tryptophan residues. Tryptophan has a high extinction coefficient that primarily contributes to the optical aborption of SA at 280 nm. Glutaraldehyde (20 mM) addition to SA caused an increased polymerization in SA (80 % from SDS-PAGE), that could have consequently decreased the protein molecules access to solvent. Our studies on the effect of pH on modified SA also supports this claim (Section 4-C).

In the case of the oxidized samples, it is most probable that the 1 nm shift in $\lambda_{\text{max}}$ observed in SA-DI at pH 7.4 could be due to changes in tryptophan environment that resulted from the intra and intermolecular contacts between spectrin chains. DI has not been shown to affect other amino acids other than cysteine, nor to cause oxidation to the sulfoxide [127]. Low concentrations of diamide form disulfides in pure spectrin [122]. From our studies, about 70 % of spectrin formed high molecular aggregates in SA-DI (1 mM) samples. Since the shift in $\lambda_{\text{max}}$ caused by spectrin polymerization was very small, minimum shielding of tryptophan residues is suggested. It is conceivable that there are no chromophores in the region of disulfide contacts. While shifts in $\lambda_{\text{max}}$ of of SA treated with $\text{H}_2\text{O}_2$ and PH could also be attributed to changes in the tryptophan environment and only to differing extent, the involvement of other tyrosine, histidine may not be discounted.

4-c. Increase in $pI$ of modified SA.

Decreasing the pH enhances the aggregation or crosslinking of pure SA [103, 133]. Large aggregates of SA are formed at pH 4.5, the isoelectric point of SA [134]. The aggregation decreases membrane spectrin solubility [134, 135]. Crosslinked SA samples are more aggregated, and are therefore expected to have decreased solubility. So studying the effect of
pH on the modified samples allow us to study the structural and aggregation (or solubility) properties of the spectrin crosslinks. Our data indicates that the modified SA samples are least soluble at their isoelectric pH. This result agrees with studies of Smith & La Celle [135] on SA solubility. One explanation to the solubility behavior of the modified sample can be inferred from the charge distribution in the SA molecule upon modification. The slight increase in the pI indicate that the crosslinked samples have more positive charge relative to non-modified SA. The highest change in pI that accompanied the modification of SA was 0.4 pH unit in SA-DI (1 mM) samples. This change, however, did not cause any major alteration in the segmental motions of SA, based on ST EPR (Section 4-f). Conventional EPR showed that about 5% of the strongly immobilized motions were converted to the weakly immobilized motions. Based on the electron micrograph studies and tryptic digestion results of Becker and co-workers [3] and our pI and EPR results, we suggest that crosslinking alters the ultrastructure, but not the local secondary structure of SA.

The pI behavior of SA-GA does not seem to be have a straightforward explanation. Modification of lysines by GA at pH 7.4 would have resulted to a SA-GA that is less negative than SA, SA-DI, SA-H2O2 and SA-PH, so in principle, the highest pI would have been be expected for SA-GA. The reversibility in GA reactions with amino acids is between 10-20% at pH 3 [107]. There is no reversibility, however, in the SA-GA adduct at pH between 6 and 8 [62]. We suspect that some reversibility in the SA-GA crosslink could have occurred upon decreasing the pH and this could have altered its positive charge distribution. The titration of some exposed residues like histidine is another possibility.

4-d. Decrease in extractibility of modified SA.

The selective release of SA is maximum at low ionic strength at 37 °C [88]. The
extractability of SA from the membrane is, therefore an indication of SA interaction with the membrane components. How this interaction is affected by the degree of crosslinking could give us information on the functional role of crosslinking in the intact membrane.

We have monitored the release of SA from the membrane using 20 mM GA-treated membranes. The GA-treated membrane samples were very resistant to the extraction of SA at low ionic strength, a condition that normally permits the selective release of SA. This indicates extensive crosslinking of SA with other membrane proteins. The integral proteins Band 3 and glycophorin that can be crosslinked, in addition to band 4.1 [86, 87]. Intact RBCs treated with H₂O₂ resulted in a 40 % decrease in extractibility of SA from the membranes [64]. Our result supplements the published [64] based on the decreased extractability of SA from oxidized membranes.

4-e. Modification in fast motions by GA, DI, H₂O₂ and PH

Mal-6 reacts with sulphydryl groups of SA (Figure 24) to give the conventional EPR spectrum shown in Figure 4. In this spectrum, the low field region exhibits two types of spin label signals: one component, W is associated with a class of weakly immobilized spin labels (w) and another component S, corresponds to a class of strongly immobilized labels (s). By spectral subtraction (Figure 12), the fast motional component can be obtained using the method of Fung and Johnson [74]. It is known that the fast component has a correlation time of 10⁻⁹ sec or faster [92]. Lammel and Maier [104] suggested that about 4-13 % of the total spectral intensity resulted from this highly mobile component, and about 10 % of the motions in spectrin-actin at pH 8 and 20 °C are included in the fast motion component [74]. The remaining majority signal (80-90 % of the total depending on sample conditions) is the S signal [94].

The amplitude ratio W/S has been used by many workers to analyze their spectra [61, 86, 92, 94, 136, 143-151]. The ratio of signal amplitudes can be used to measure the relative
Figure 24. The reaction of 4 Maleimide-2,2,6,6-tetramethyl-1-piperidinyloxy free radical (Mal-6) protein sulphydryl groups (Griffith and McConnell, 1966). Spin labeling of ghosts with this radical was carried out with 30-50 µg of Mal-6/mg protein as described in Materials and Methods.
amounts of w in the system. Since W exhibits a relatively sharp line, minor changes in this weakly immobilized motion produce significant changes in the W/S value. For example, a 5% change in the w component gives about a W/S change of 60% at pH 8 and 20 °C [136]. Thus, W/S ratios do not necessarily indicate very different amounts of the W component in the corresponding samples. At pH 8 and 20 °C, 10% W gives a W/S value of about 4 for membrane samples [136]. The W/S ratio and thus, the fast motion component W, is very sensitive to pH, T, µ, and the presence of divalent cations (Mg and Ca ions) for both membrane and SA samples. Small alterations in the physical state of the membrane that are caused by the changes in temperature or pH of the sample, will only cause minor changes in the motions of the membrane proteins [74, 104, 105].

Our studies using isolated SA treated with 20 mM GA showed increased W/S values, (Figure 9a), a result that was not expected. To see whether the results observed were not due to other artifacts, the W/S trend for the membranes treated with different concentrations was obtained. The decreasing W/S of the ghosts indicate restricted mobility which may be attributed to extensive crosslinking in the membrane of SA, band 3 and glycophorin. Band 3 is anchored to SA by the protein ankyrin. Crosslinking of SA and the integral proteins in the membrane presumably triggered the decrease in the W/S value. This result provides evidence that the increased W/S in SA-GA samples was due to crosslinking. The influence of protein-lipids interactions on the fast motional component of GA-treated membranes is therefore indicated.

Measurements of the viscosity [137], the sedimentation coefficient [138] and light scattering [139] indicate that at low ionic strength, spectrin undergoes a slight expansion and an increase in contour length. On the other hand, spectrin-actin contracts in the presence of 30 mM to 300 mM NaCl [138] which is consistent with a decrease in the hydrodynamic radius of spectrin is observed when at NaCl concentrations greater than 20 mM [88, 139]. These expansion and
contraction of membrane skeletons which are mainly spectrin-actin upon change of ionic strength have recently been confirmed [140] and are related to spectrin self-association [149]. So the question is whether these structural modifications in SA triggered by changes in ionic strength are accompanied by a loss of motional flexibility in the GA-treated SA. We have used the W/S ratio to monitor the motional response of SA-GA samples to high salt. The 25% decrease in the W/S that we observed for SA-GA (20 mM) samples in the presence of high salt (Figure 9b) could indicate only a slight, restricted motion due to salt-induced structural alterations in the SA skeletal network. It is known that W/S of SA decreases as NaCl is increased [136]. It is probable that the increased association to the tetramer and higher oligomers at ionic strength greater than 50 mM [142, 143] could have affected the ionic interactions in the SA molecules resulting to slightly slower motion. This decrease in W/S is even made more significant by the increase polymerization due crosslinking of SA by GA.

A more useful parameter than the absolute value of W/S ratio for studying membrane interactions is $\Delta(W/S)$ [145]. $\Delta(W/S)$ is the difference between W/S ratios in the presence and absence of crosslinker. It is known that this difference parameter eliminates most of the variations due to different membrane preparations, permitting more sensitive detection of the effects of extrinsic agents upon membrane proteins [86, 135]. We have used the change in W/S ratio versus to get more quantitative information about the crosslinker and SA interaction (Figure 6). The change in W/S fraction is related to the amount of spin labeled sites in the protein that have the crosslinker associated with them (see Materials and Methods). In terms of a simple two-state model hypothesis [132], the results indicate that crosslinking caused a slight increase in the mobility of the modified region at pH 7.4.

Lowering the pH is another way to monitor the motional effects of crosslinking on SA (Figure 7). It is known that the erythrocyte membrane shrinks upon decreasing the pH to form
aggregates at pH 4.5, the isoelectric point of SA [142]. Pure spectrin-actin also form similar aggregates at pH 4.5 [134] and at low pH, spectrin tetramer formation is favored [141]. The little or no significant difference in the hyperfine separations and line widths of the hyperfine extremal lines of the conventional EPR spectra at 20 °C and at low pH indicate similar mobilities. The crosslinked samples appeared to be strongly immobilized on the conventional EPR time scale (τ > 10⁻⁷ sec) at their isoelectric points. The W/S of SA modified with GA (20 mM), DI (1 mM), H₂O₂ (0.5 mM) and PH (20 mM) at pH 7.4 were 3.45 ± 0.07 (n=18), 3.90 ± 0.10 (n=3), 3.74 ± 0.05 (n=3) and 2.70 ± 0.05 (n=3), respectively. By lowering the pH from 7.4 to the individual pI values, the fast motional components were presumably converted to slow motional components.

4-f. Modifications in slow motions by GA, DI, H₂O₂ and PH

The majority of the EPR spectral intensity is associated with the strongly immobilized signal s, and conventional EPR detection is inadequate to study motional behavior of this component due to its insensitivity to motions slower than 10⁻⁷ sec. To study molecular motions with rotational correlation times of 10⁻⁶ - 10⁻³ sec, saturation transfer (ST) EPR is used [95, 152, 153]. The spectral amplitudes at the diagnostic field positions of the ST-EPR spectra are very sensitive indicators of rotational motions and characteristic ratios of spectral intensities can be obtained. These spectral ratios L''/L, C'/C, and H''/H from the diagnostic regions of the ST-EPR spectrum and correspond to the low, center and high field regions, respectively. In each ratio, the numerator is the line height that is most sensitive to motion with a spectral region, and the denominator is the least sensitive [95]. These ratios significantly change upon decreasing the temperature from 20 - 5 °C for both pH 8 and pH 4.5 [92].
Similar ratios were observed for SA and oxidized SA indicating similarity in segmental motions (Tables 3-6). The values were consistent with the superimposition of slow and very slow motions in SA characterized by Fung and Johnson [74]. SA possesses considerable segmental flexibility [41, 79, 101]. A change in this flexibility due to crosslinking may be expected. However, moderate crosslinking of SA did not cause significant changes in the segmental motions of SA.

The signal amplitude ratios can be defined from the ST-EPR spectra as a function of rotational correlation times [95]. $\tau_H$, $\tau_C$, or $\tau_L$ is correlation time from either low, central, or high field region for a system exhibiting isotropic motion. If a molecule, for example, has different rotational correlation times from the three ratios, this means that the molecule is exhibiting anisotropic motions [152, 154] or a superimposition of motions [74]. Correlation times have been obtained by ST-EPR to study the motions and dynamics of various spin labeled proteins [74, 95, 152-161].

Using X-band ST-EPR, correlation times of SA have been reported at pH 4.5 [85]. At 20 °C, $\tau_H = 6 \times 10^{-3}$ sec, $\tau_L = 4 \times 10^{-4}$ sec, and $\tau_C = 4 \times 10^6$ s. At 10 °C, $\tau_H$ and $\tau_L = 10^{-3}$ s, and $\tau_C = 7 \times 10^4$ s. At 0 °C, $\tau_H$ and $\tau_L > 10^8$ s and $\tau_C = 1 \times 10^{-3}$ s. Since the $\tau_H$, $\tau_C$, and $\tau_L$ values obtained from the same spectrum are different, this indicates that the spectrin-actin motion is anisotropic and/or a superposition of different motions. Studies using Q-band ST-EPR show that the motion observed in SA is not consistent with simple isotropic motion nor two types of axial anisotropic motion. The spectral features obtained were consistent with superimposition of slow and very slow motions having by correlation times $10^{-7}$ sec and $10^{-3}$ sec [74].

Rotational correlation times of spectrin obtained from other methods under different conditions have been reported [74, 77, 78, 79, 80, 81]. We have obtained the rotational correlation times from the slow motion ST-EPR spectra of SA modified with GA and DI
(Tables 3 and 5). After spectral subtraction of the fast motional component, the resulting slow motion ST-EPR spectra (Figure 14) of the modified samples suggest correlation times of $10^{-7}$ sec from $C'/C$ and about $10^{-5}$ sec from the $L''/L$ and $H''/H$ ratios. A comparison of the $\tau_H$, $\tau_C$, or $\tau_L$ for SA, SA-GA (20 mM) and SA-DI (1 mM) at pH 8 and 20 °C indicate no detectable change in the segmental motions of SA. A similar conclusion was reached by comparing the $\tau_H$, $\tau_C$, or $\tau_L$ for SA, SA-GA (20 mM) and SA-DI (1 mM) at pH 6 and 20 °C.

The ST EPR spectra at pH 6 and 4 °C showed mostly strongly immobilized motions so spectral subtraction was not necessary. The rotational correlation times in square brackets [ ] obtained from the composite spectra of SA modified with GA, DI, $H_2O_2$ or PH at pH 6 and 4 °C as shown in Table 6 were similar, suggesting that modification did not significantly change the conformational flexibility of SA even at saturating levels of the reagents.

Some parameters correlations can be drawn. The UV data at pH 7.4 showed an 8 nm shift in $\lambda_{\text{max}}$ for SA-GA compared to 1-3 nm $\lambda_{\text{max}}$ SA-DI, SA-$H_2O_2$ or PH indicating that modification of SA by 20 mM GA had effect the most changes in the protein’s chromophoric groups. This result is consistent with the highest degree of crosslinking for SA-GA samples. GA-modified membranes were resistant to SA extraction suggesting that the degree of crosslinking caused by GA is extensive. This observation supplements the findings of Sauberman et. al. [64] that RBCs oxidized with $H_2O_2$ produce low yield of SA. The overall pI data agrees with the higher degree of crosslinking of SA at low pH. Previous studies of SA showed that SA aggregation is indeed enhanced at low pH [126, 127, 128]. Taken all together, a common effect on the segmental motions of spectrin by the degree of crosslinking is operating, even if the crosslinkers we used differ in mechanistic details.
4-g. **Summary**

From these studies, we have demonstrated that crosslinking SA, either at lysine or cystein residues, to form high molecular weight complexes did not lead to motional restriction in SA molecules. The use of conventional and ST-EPR allowed us to monitor the complex motions of SA and SA modified with GA, DI, H₂O₂ or PH. Fung and Johnson [74] reported that these complex motions which are classified as fast, slow and very slow motions, have rotational correlation times of about $10^{-9}$ sec, $10^{-7}$ sec, and $10^{-3}$ sec respectively. The relative proportion of the slow and very slow motions is moderately sensitive to temperature over the range of pH 6 to pH 8.

The EPR probe detects the local motion, the local molecular environment, the local SA dynamics from milliseconds to picoseconds. The segmental motions detected in these studies are motions along the flexible loops of SA and modified SA molecules, and not necessarily reflective of the overall motion of the protein. Our studies provide no evidence that these complex segmental motions have been restricted by the degree of oxidation of SA. Substantial segmental motions remains after SA modification, indicating that the conformational flexibility of the isolated SA molecule and the degree of crosslinking are molecular properties that are mutually exclusive. It is known that attaching SA to membrane components does not lead to immobilization of SA [85, 75, 76]. Our results are consistent with these studies.

Our results are also consistent with the complex or superposition of motions in SA and modified SA samples. Variations within the same modified system could be due to two reasons: (1) the crosslinking sites for a particular system may not have been the exactly the same each time a sample is prepared even under the same conditions, or (2) the different line height ratios have different sensitivities to the presence of even a small fast component that would not have been completely removed by spectral subtraction. Isoelectric pH and UV studies indicate
been completely removed by spectral subtraction. Isoelectric pH and UV studies indicate changing surface topography and overall charge distribution of the oxidized SA molecules, as well as the reaction capabilities of the aromatic residues, in addition to the state of SA aggregation. Under the conditions used, these studies show that there is no pronounced relationship between structural modification and segmental flexibility in SA. In our motional studies, the specific location of the labeling and crosslinking sites involved in changing the segmental motions were not precisely identified. The determination of these sites and the site-specific attachment of the spin labels should lead to a more meaningful characterization of the conformational flexibility and local dynamic processes of SA molecules.
APPENDIX
Appendix A. The Stability of Mal-6 spin label

The spin label used in this experiment is N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (Mal-6). Mal-6 belongs to a group of free radicals called nitroxides. Nitroxides are organic free-radical reporter groups that can be chemically attached to strategic points on biomolecules to provide information on the local molecular environment. Nitroxide radicals have unpaired electron density located mainly in the oxygen and nitrogen orbitals, and are very stable [162].

The high kinetic stability of the piperidinyl nitroxide radicals (where the four methyl substituents screen the radical center and of which Mal-6 is an example) is accounted for by the low level of the ground state energy of the system with three electron nitrogen-oxygen bond [163], and also by the considerably high steric factor in the region of the free valence localization [162, 164]. The triplet nature of the EPR spectrum accounted for by the interaction between the magnetic moment of the unpaired electron and by that of the nitrogen nucleus of a spin equal to unity, is an indication that considerable amount of localization of the unpaired electron cloud occurs in the heteroatom [164, 165]. The functional group attached to the other end of the piperidinyl ring, therefore, enters into reactions without involving the free valence. This accounts for the remarkable stability and inertness of the nitroxide group in the Mal-6 spin label.
REFERENCES


Vita

The author, Benito Manolo O. Kalaw, was born on January 12, 1956 in Batangas City, Philippines. He attended Libjo Elementary School and Western Philippine Colleges’ High School. He went to college at the University of the Philippines (U.P.) in Quezon City, Metro Manila. While at the U.P., he was sent to Canada by the Philippine Government as an exchange student in 1976 and became the President of the U.P. Chemical Society in 1979. He obtained a BS Chemistry degree from the U.P. in 1981.

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April 18, 1994  
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