Erythrocyte Spectrin Maintains Its Segmental Motions on Oxidation: A Spin-Label EPR Study

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ABSTRACT The segmental motions of cross-linked erythrocyte skeletal protein (spectrin–actin–protein 4.1) samples, labeled with nitroxide spin labels, were monitored by conventional first-harmonic and saturation transfer second-harmonic electron paramagnetic resonance methods. Skeletal proteins were extracted from human red blood cells and treated with three oxidative reagents (diamide, hydrogen peroxide, and phenylhydrazine) to cross-link sulfhydryl groups and with one fixative reagent (glutaraldehyde) to cross-link lysine residues. The treatments provided extensive cross-linking between spectrin–actin–protein 4.1 molecules, as determined by gel electrophoresis, and surface charge modification, as determined by pI measurements. However, segmental motions of the cross-linked skeletal proteins remained generally similar to those in normal skeletal proteins. Both the weakly immobilized and the strongly immobilized motions were similar in cross-linked and control samples. Small differences in some motional components were detected. In some cases, faster mobilities were observed, with ~5% of the strongly immobilized motions converted to the weakly immobilized motions in the cross-linked samples. It is often believed that the consequence of membrane protein oxidation is restricted protein dynamics, giving membrane rigidity. However, our studies provide needed experimental evidence to indicate that segmental motions are maintained with very little modification even in the presence of extensive cross-linking. Thus cross-linking does not restrict the internal molecular flexibility that gives rise to segmental motions.

INTRODUCTION

The survival of human erythrocytes depends on their deformability and elasticity, which are governed, in part if not solely, by the cell membrane's underlying protein skeleton. The mechanism by which this skeletal network controls deformability and elasticity is not clear (Mohandas, 1992; Nash and Gratzer, 1993; Mohandas and Evans, 1994). Oxidants can alter membrane properties, produce rheological impairments, and cause ultimate hemolysis in human erythrocytes. The molecular events responsible for these changes are also not well understood (Becker et al., 1986). At the membrane level, cellular rigidity in oxidized erythrocytes has been associated with increased membrane rigidity (Hochstein and Jain, 1981; Hebbel et al., 1990) or decreased membrane fluidity (Rice-Evans and Hochstein, 1981). At the protein level, cellular rigidity in oxidized erythrocytes has been associated with cross-linked membrane proteins, especially spectrin, forming high molecular weight complexes (HMWCs) (Haest et al., 1977; Kosower et al., 1981; Maeda et al., 1983; Vilsen and Nielsen, 1984; Becker et al., 1986). Circular dimers or single chains were detected in diamide-treated spectrin; however, no difference in tryptic digestion pattern was observed in oxidized spectrin (Becker et al., 1986). The authors suggested that oxidation produces local secondary conformational changes in spectrin. Thus it is possible that oxidation leads to changes in molecular (structural and/or dynamic) properties of spectrin, which lead to membrane rigidity and then to reduced cell deformability. However, experimental evidence linking cell deformability properties to molecular components remains scarce.

Native spectrin heterodimer molecules exhibit considerable segmental motion. Our early spin-label electron paramagnetic resonance (EPR) studies showed three principal motional components with correlation times of $\leq 10^{-9}$, 10^{-7}-10^{-6}, and $\sim 10^{-3}$ s (Fung and Johnson, 1983). 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 (Lemaigre-Dubreuil and Cassoly, 1983). 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 (Calvert et al., 1980; Fung et al., 1986, 1989; Begg et al., 1994), with 10–15% of the total protons in spectrin exhibiting rapid motions (Fung et al., 1989). Phosphorescence anisotropy studies at 10°C give correlation times of 3×10^{-6} s for the spectrin dimer and tetramer and also 30×10^{-6} s for the tetramer (Learmonth et al., 1989), whereas transient dichroism studies gave correlation times of $\sim 10^{-4}$ s at 4°C (Clague et al., 1990). Dynamic light scattering measurements supply further evidence for fluctuational segmental motions of spectrin occurring over a

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Abbreviations used: EPR, electron paramagnetic resonance; HMWCs, high molecular weight complexes; Mal-6, *N*-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidinyl) maleimide; NMR, nuclear magnetic resonance; pI, isoelectric pH; S, signal amplitude of the low field region of the strongly immobilized motion; SDS, sodium dodecyl sulfate; SH, sulfhydryl; ST, saturation transfer; VHMWCs, very high molecular weight complexes; *W*, signal amplitude of the low field region of the weakly immobilized motion.

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relatively large distance of 22–30 nm with relaxation times of $\leq 23 \times 10^{-6}$ s (Budzynski et al., 1992).

It is not clear whether oxidation restricts segmental motions in spectrin. No experimental evidence correlates oxidation or membrane rigidity with spectrin segmental motions, although it is assumed that constraints on the freedom of movement of the spectrin skeleton relative to the membrane have a major influence on cell rigidity (Nash and Gratzer, 1993). We used spin-labeling methods to monitor spectrin segmental motions in samples treated with different cross-linking reagents. Diazine dicarboxylic acid (bis[*N*, *N*-dimethylamide]; diamide), hydrogen peroxide, phenylhydrazine (all considered oxidants), and glutaraldehyde (a fixative reagent) were used to cross-link spectrin and other proteins in the skeletal protein samples. We found a concentration-dependent formation of protein complexes, as expected. We also detected surface charge modification. However, the cross-linking did not appear to reduce segmental motions in spectrin. The majority of the motions in modified samples remained similar to those in unmodified samples. Very slight increases in the proportion of weakly immobilized components were detected in cross-linked samples.

MATERIALS AND METHODS

Preparation of skeletal proteins

Human red blood cells were obtained from the local blood bank. All procedures were carried out at 4°C. Packed cells were gently washed with 5-mM sodium phosphate buffer containing 150-mM NaCl at pH 7.4. Membranes were prepared from washed red blood cells with 5-mM phosphate buffer at pH 7.4 (Fung, 1981). Protein concentrations in membranes were determined by the modified Lowry method (Larson et al., 1986) and were then adjusted to 4 mg/ml (~7 μM of spectrin dimer, using spectrin molecular mass of 550 kDa to estimate the molar concentration because spectrin was the major protein in the skeletal protein sample). The membranes were then spin labeled with *N*-(1-oxyl-2, 2, 6, 6-tetramethyl 4-piperidinyl) maleimide (Mal-6; Aldrich, Milwaukee, WI) at 120–200 μg/ml membrane proteins (480–800 μM of Mal-6) in the dark for 1 h (Fung and Johnson, 1983). Excess Mal-6 was removed by washing with buffer until the samples gave constant EPR signals. The spin-labeled skeletal protein sample was extracted from spin-labeled membranes with 0.3-mM phosphate buffer at pH 7.6 at 37°C (Bennett and Branton, 1977). The spin-labeled skeletal protein sample was concentrated in an ultrafiltration cell (Amicon, with YM-100 membranes), followed by dialysis with appropriate buffer. The extinction coefficient of 10 for 1% (10 mg/ml) sample (Bennett and Branton, 1977) was used to determine protein concentrations. As indicated below, we also determined the extinction coefficient of our samples and obtained a mean value of 11 for 1% skeletal protein samples.

The skeletal protein sample without spin labels was prepared similarly, except for omission of those steps involving spin labeling. Spin labeling did not affect the amount of skeletal proteins extracted from intact membranes. Because spin labeling may perturb skeletal protein properties, this extraction result provides some reassurance that the spin-labeled skeletal protein sample prepared from labeled membranes resembles the nonlabeled (native) skeletal protein sample.

Glutaraldehyde, diamide, hydrogen peroxide, and phenylhydrazine modifications

The spin-labeled skeletal protein sample (~2 mg/ml) in 5-mM phosphate buffer at pH 7.4 was treated with various amounts of glutaraldehyde (Grade I, 25% aqueous solution from Sigma) for 20 min at 22°C (Steck, 1972), diamide for 60 min at 37°C (Kurantsin-Mills and Lessin, 1981), phenylhydrazine for 60 min at 37°C (Arduini et al., 1986), or hydrogen peroxide for 15 min at 37°C (Snyder et al., 1985). After the incubation period, the mixtures were either mixed with sodium dodecyl sulfate (SDS) solution for electrophoresis studies or dialyzed and concentrated to ~10 mg/ml by vacuum dialysis (Prodicon, Danvers, MA) at 4°C for EPR studies. Extinction coefficients for these samples at 280 nm were determined (with protein concentrations obtained from modified Lowry method) such that protein concentrations could be determined conveniently. The extinction coefficients for 1% samples at 280 nm were 11.0 ± 0.7 for nonmodified skeletal protein samples, 14.9 ± 0.1 for glutaraldehyde (20 mM) treated samples, 12.3 ± 0.1 for diamide (1 mM) treated samples, 12.8 ± 0.1 for hydrogen peroxide (0.5 mM) treated samples, and 11.5 for phenylhydrazine (20 mM) treated samples.

We checked the purity of glutaraldehyde before use by scanning between 230 and 300 nm, as pure glutaraldehyde absorbs at 280 nm.

Electrophoresis

SDS polyacrylamide (7% acrylamide) gel electrophoresis was carried out in the presence of dithiothreitol on skeletal protein samples treated with glutaraldehyde (Steck, 1972) and in the absence of dithiothreitol on samples treated with diamide, hydrogen peroxide, or phenylhydrazine (Becker et al., 1986). Control samples were skeletal protein samples with and without dithiothreitol. Approximately 8 μg (10 μl) of proteins were used for each lane. Protein bands were stained with Coomassie Blue R250. An image scanner (Hewlett Packard HP ScanJet 3p) was used to produce gel images, which were digitized (Sigma Scan/Image, Jandel) into intensities as a function of distance for each lane. Each lane was divided into two sections with the dividing line at a position immediately above band 1 (the position occurred at a distance = 37 from the top edge of the gel, with a total gel length of 280): the first section was the part of the gel above the 37 line (top part of the gel), and the second section was below the 37 line (bottom part of the gel) (see Fig. 3 below). We obtained integrated intensities of peaks in the top and bottom parts of the gel by using the computer software, Origin (Microcal Software, Inc.). The integrated intensities in the top part of the gel were assumed to be from cross-linked proteins (HMWCs). Integrated intensities in the bottom part of the gel were assumed to be from the remaining nonmodified proteins in samples treated with cross-linking reagents. We compared total integrated intensities (sum of the intensities of the top and bottom parts of the gel) of cross-linked samples with control samples (with or without dithiothreitol) to determine amounts of proteins that were too large to enter the gel and remained on the top edge of the gel.

pI studies

The pH values of spin-labeled samples at 1 mg/ml in 5-mM phosphate buffer at pH 7.4 were adjusted with 1-N HCl at 22°C for the determination of isoelectric pH (pI) values. As pH values of samples decreased to ~5.5 or below, precipitation was observed in the samples. Samples were then centrifuged at ~500 g. We obtained absorbance values at 280 nm for all the supernatant for concentration determination, using the extinction coefficients discussed above. pH values and concentrations were plotted. The pH value corresponding to minimal concentration of each sample was taken as the pI point of that sample. Third-order polynomial functions provided by the Origin software were used to group data points of each sample for ease of reading and had no theoretical significance.

EPR

EPR experiments were performed on an x-band spectrometer (Varian Associates, Palo Alto, CA) equipped with a variable-temperature unit (IBM Instruments, Inc., Danbury, CT). The spectrometer was interfaced to

a PC for data acquisition and data analysis. EPR samples were introduced into 50-μl capillary tubes. Conventional first-harmonic and saturation transfer (ST) second-harmonic spectra were obtained with procedures established in our laboratory (Fung, 1981; Fung and Johnson, 1983).

Spin-label concentrations were obtained from spectra after double integration. Approximately 2×10^{-4} M of Mal-6 was found in a 10-mg/ml skeletal protein sample. The average number of Mal-6 per spectrin molecule was 13 ± 1 ($n = 10$), with 550 used as the molecular weight for spectrin in the calculation. There are 35 sulfhydryl (SH) groups (cysteine residues) in spectrin dimer (Sahr et al., 1990; Winkelmann et al., 1990). Thus 30–40% of the total SH groups were alkylated with spin labels in the samples that we used.

Conventional EPR spectral analysis

The spectra of labeled skeletal protein samples (Fig. 4 below) consist of overlapping components: one set of narrow lines for weakly immobilized motions and one set of broad lines for strongly immobilized motions, with the corresponding signal amplitudes denoted W and S, as reported before (Fung, 1983). The W/S ratios of the unmodified and modified samples were calculated. Spectral subtraction methods used previously (Fung and Johnson, 1983) were carried out to resolve the composite spectrum of weakly and strongly immobilized components into two single-component spectra. Briefly, the proportion of the fast component is quite sensitive to pH over the range pH 6–8 (Fung and Johnson, 1983). The spectrum of skeletal protein sample at pH 8 and 20°C was subtracted from the spectrum at pH 6 and 20°C to give a difference spectrum, and this difference spectrum was used as the weakly immobilized component spectrum for all samples in a second spectral subtraction to remove the weakly immobilized component in spectra consisting of both strongly and weakly immobilized components. The resulting spectra (the strongly immobilized spectra) and the original composite spectra were integrated to provide the proportions of weakly and strongly immobilized components in each sample. If the weakly immobilized component in the modified samples differed from that in the control samples (e.g., different linewidths), then the weakly immobilized component in the modified sample would not be removed by these spectral subtraction methods.

Saturation transfer EPR spectral analysis

Inasmuch as most of the spectra consisted of weakly and strongly immobilized components, again spectral subtraction similar to the method used for conventional EPR spectral analysis discussed above was necessary to give spectra of only strongly immobilized motions. Signal averaging to give spectra with relatively high signal-to-noise ratios were needed to provide reliable spectral subtraction. The spectral amplitudes L and L'' in the low-field region, C and C' in the central region, and H and H'' in the high-field region in ST EPR spectra (Thomas et al., 1976) were measured, and the L''/L, C'/C, and H''/H ratios were calculated. We obtained apparent rotational correlation times from these ratios, using published calibration curves (Thomas et al., 1976). Experimental uncertainties of these measurements were determined from standard error of the mean (SE).

We also used two different analyses to evaluate the apparent rotational correlation times, as detected by ST EPR methods, to determine whether the motional components of control and modified systems were, within experimental uncertainties, statistically different. The first method that we used was paired Student's t-tests on individual τ values of control and modified samples under specific experimental conditions. The second method that we used was a null-hypothesis Student's t-test on $\log[\tau$ (modified)$/\tau$ (control)] for the full data set. Student's t-tests were performed with commercially available statistical software (Systat 5.0; Systat, Inc., Evanston, IL).

RESULTS

Extent of protein cross-linking in skeletal proteins by glutaraldehyde and by diamide, hydrogen peroxide, and phenylhydrazine

Skeletal proteins extracted from membrane ghosts consisted mostly of spectrin (bands 1 and 2) (Fig. 1, lanes 2 and 7; Fig. 2, top two curves). Also observable in gels of skeletal protein samples, especially those carried out in the presence of dithiothreitol (Fig. 1, lane 2; Fig. 2, middle curve), were protein 4.1 (band 4.1) and actin (band 5). Because oxidation in the skeletal proteins involves SH cross-linking, we performed gel electrophoresis without dithiothreitol on samples treated with oxidants (diamide, hydrogen peroxide, and phenylhydrazine), using skeletal protein samples without dithiothreitol as controls. For a typical skeletal protein sample in the absence of dithiothreitol, the integrated intensity above band 1 in the top part of the gel (see Electrophoresis in the Methods section for a definition of "top part") was 1637 and the intensity below band 1 in the bottom part of the gel was 4084 (Fig. 3, right-hand panel, dotted curve). No protein was observed on the top edge of the gel. Thus ~30% of the proteins in skeletal protein samples in the absence of exogenous oxidants were involved in SH cross-linking to form HMWCs. However, the cross-linking did not appear to be extensive because the positions of the bands were only slightly above band 1 (Figs. 1 and 3). The uncertainties of

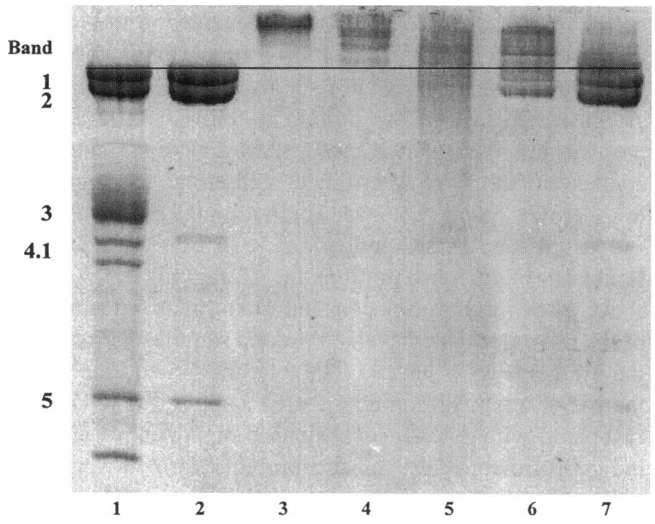

FIGURE 1 Electrophoresis results of SDS polyacrylamide gel (7%) of skeletal protein samples with and without cross-linking reagents. *Lane 1*, Membrane ghosts; *lane 2*, skeletal proteins; *lane 3*, skeletal proteins treated with 20-mM glutaraldehyde. Samples in lanes 1–3 were taken in the presence of dithiothreitol. *Lane 4*, Skeletal proteins treated with 1-mM diamide; *lane 5*, skeletal proteins treated with 20-mM phenylhydrazine; *lane 6*, skeletal proteins treated with 0.5-mM hydrogen peroxide; *lane 7*, skeletal proteins. Samples in lanes 3–7 were taken in the absence of dithiothreitol. Approximately 8 μg (10 μl) of proteins were used for each lane. Protein bands were stained with Coomassie Blue R250. An image scanner (Hewlett Packard HP ScanJet 3p) was used to produce the gel images presented. The solid line above band 1 and across all lanes corresponds to the "37 line" in Fig. 3 below.

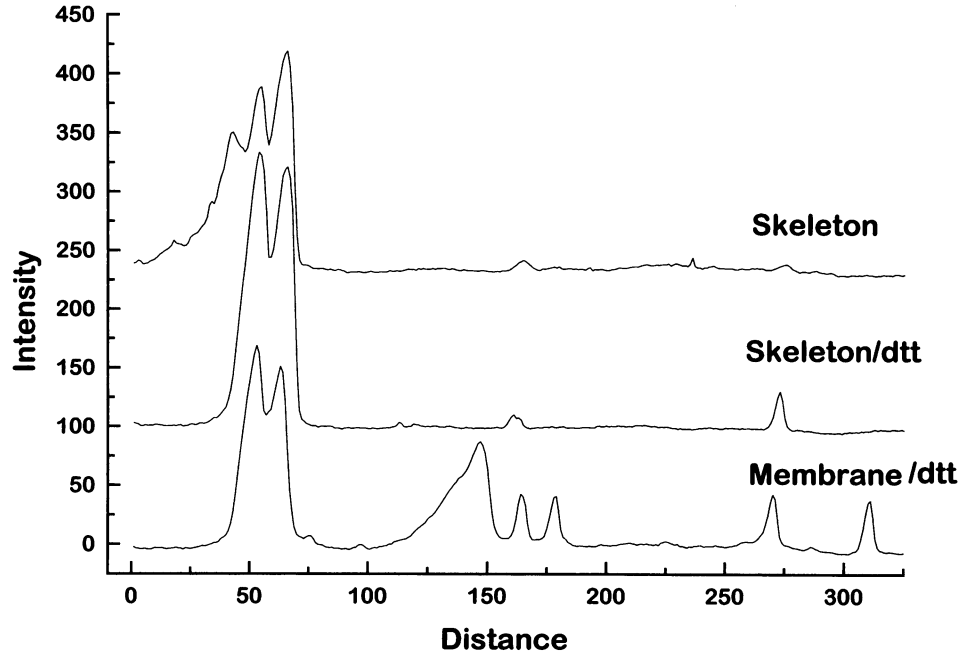

FIGURE 2 Digitized gel images of lanes 1 (membrane ghosts with dithiothreitol), 2 (skeletal proteins with dithiothreitol), and 7 (skeletal proteins without dithiothreitol) of Fig. 1. Gel images were digitized into intensities as a function of distance with the Sigma Scan/Image software from Jandel.

our intensity measurements were generally less than 10%. The variations that resulted from sample loading between different lanes on the same gel or between different gels were also usually less than 10%.

As concentrations of oxidants in skeletal protein samples increased, the integrated intensities in the bottom part of the gel decreased, and the integrated intensities in the top part increased accordingly (data not shown), until saturating concentrations were reached. Thus the decrease in integrated intensities in the bottom part of the gel and the increase in the top part reached plateau values at saturating concentrations. We determined saturating concentrations for diamide to be ~1 mM, for hydrogen peroxide to be ~0.5 mM, and for phenylhydrazine to be ~20 mM, with skeletal protein concentrations of ~2 mg/ml.

At or above saturating concentrations, approximately half of the diamide-treated skeletal protein samples did not enter the 7% gel (Fig. 1, lane 4). The total integrated intensity on the gel of a typical diamide-treated sample was only 2343, compared with 5721 for a skeletal protein sample (Fig. 3), indicating that ~59% of the proteins formed very high molecular weight complexes (VHMWCs) and did not enter the gel. Of those that did enter the gel, ~10% remained as spectrin–actin–protein 4.1 (with an integrated intensity in the bottom part of 572) and ~31% (with an integrated intensity in the top part of 1771) were converted to HMWCs (Fig. 3, bottom curves). These results as well as those for the samples treated with hydrogen peroxide, phenylhydrazine, and glutaraldehyde, at saturating concentrations, and their controls are summarized in Table 1. Thus, under our experimental conditions, glutaraldehyde and diamide at saturating concentrations cross-linked 90% or more of the skeletal proteins, each with a different mechanism, and produced different complexes, as expected. Diamide, a bifunctional SH reagent, has been shown to oxidize stoichiometrically the SH group of cysteine in proteins to form disulfide bonds without generating radicals (Kosower et al., 1969). Glutaraldehyde reacts primarily with the ϵ-NH_2 group of lysine to form intermolecular cross-linkages (Habeeb and Hiramoto, 1968). Hydrogen peroxide oxidation of SH groups in proteins involves radical reactions (Little and O'Brien, 1969). Oxidation products of phenylhydrazine, including hydrogen peroxide, are responsible for membrane protein oxidation (Misra and Fridovich, 1976; Chakrabarti et al., 1995). On addition of hydrogen peroxide and phenylhydrazine, ~50% of the skeletal proteins cross-linked and formed probably another set of cross-linked complexes. These oxidation results for spin-labeled skeletal proteins (with 30–40% of the SH sites being labeled with spin labels) are quite similar to results obtained for samples without spin labels.

pI values

The pI values of the modified samples were all slightly higher than the pI value of the control sample. In a typical run, the pI value of the control sample was 4.44, and values were 4.70, 4.77, 4.83, and 4.90 for samples treated with phenylhydrazine, glutaraldehyde, hydrogen peroxide, and diamide, respectively (Fig. 4). The average pI value, with $n = 3$, was 4.47 ± 0.03 for skeletal protein samples. The average pI values were 4.75 ± 0.03 for samples treated with 20-mM phenylhydrazine, 4.81 ± 0.01 for samples treated with 20-mM glutaraldehyde, 4.84 ± 0.01 for samples treated with 0.5-mM hydrogen peroxide, and 4.91 ± 0.08 for samples treated with 1-mM diamide. Thus, proteins in different modified skeletal protein samples exhibit net charges different from those in the nonmodified skeletal

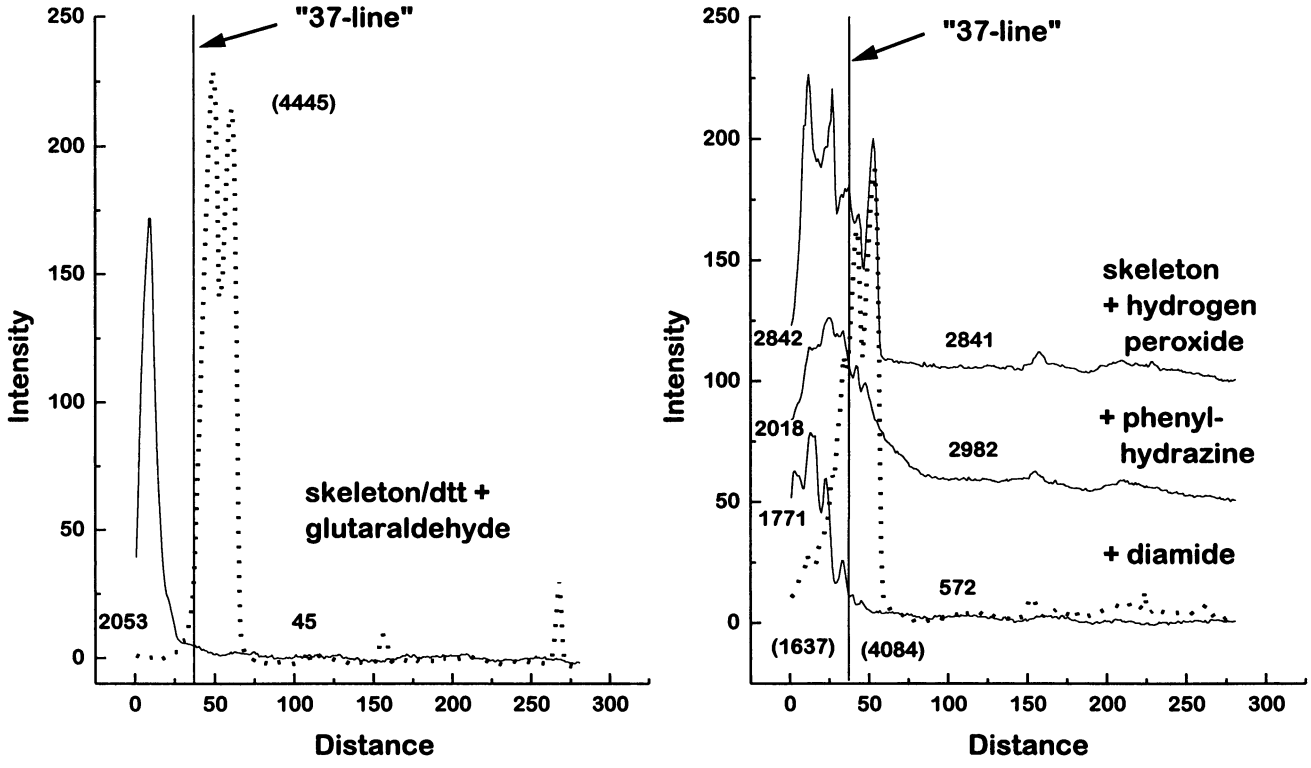

FIGURE 3 Digitized gel images of cross-linked skeletal proteins and their integrated intensity values (obtained with the Origin program from Microcal Software, Inc.). The integrated intensity measurements of each lane were divided into two sections with the dividing line at the top of band 1 position (distance = 37 from the top of the gel): section 1 was above the 37 line (top part of the gel), and section 2 was below the 37 line (bottom part of the gel). Integrated intensities of the top part were assumed to be from cross-linked proteins (HMWCs) and of the bottom part were assumed to be from the remaining nonmodified proteins in samples treated with cross-linking reagents. *Left panel*: Lane 3 of Fig. 1 (sample treated with glutaraldehyde in the presence of dithiothreitol): The integrated intensity was 2053 for the top part and 45 for the bottom part. The dotted curve is that for skeletal proteins with dithiothreitol and is shown for comparison. The integrated intensity of the control sample (*dotted curve*) was 4445 for the bottom part. *Right panel*: *Top*, lane 6 of Fig. 1; sample treated with hydrogen peroxide in the absence of dithiothreitol. The integrated intensity was 2842 for the top part and 2841 for the bottom part. *Middle*, lane 5 of Fig. 1; sample treated with phenylhydrazine. The integrated intensity was 2018 for the top part and 2982 for the bottom part. *Bottom*, lane 4 of Fig. 1; sample treated with diamide. The integrated intensity was 1771 for the top part and 572 for the bottom part. The dotted curve is that for the control sample (skeletal proteins) and is shown for comparison, with the integrated intensity being 1637 for the top part and 4084 for the bottom part.

protein samples. These data supported the gel electrophoresis results, indicating that the skeletal proteins were modified by oxidants or cross-linking reagents.

W/S ratios

Conventional EPR spectra of spin-labeled skeletal protein samples (controls) in 5-mM phosphate buffer at pH 7.4 and 20°C exhibited weakly and strongly immobilized components (Fig. 5a) with average *W/S* values of 2.39 ± 0.03 (n = 18), in good agreement with published values (Fung and Johnson, 1983). Most of the spin labels in the skeletal protein are on spectrin (Fung and Simpson, 1979). It was interesting, as well as surprising, to find slightly more weakly immobilized components in samples treated with either glutaraldehyde or oxidants than in the control sample (Fig. 5). For all samples treated with cross-linking reagents, *W/S* values increased proportionally as concentrations of cross-linking reagents increased (Fig. 6). Samples treated with diamide (Fig. 6 *B*) or phenylhydrazine (Fig. 6 *D*) exhibited the largest increases in *W/S* values, whereas samples treated with hydrogen peroxide exhibited only slight increases (Fig. 6 *C*). The average *W/S* value was 3.45 ± 0.07 (n = 11) for samples treated with glutaraldehyde at saturating concentration, 3.90 ± 0.02 (n = 4) for samples treated with diamide, 2.65 ± 0.02 (n = 4) for samples treated with hydrogen peroxide, and 3.75 ± 0.05 (n = 3) for samples treated with phenylhydrazine.

Because the weakly immobilized signal is a narrow signal, whereas the strongly immobilized signal is a broad signal, a large increase in the amplitude ratio *W/S* may represent only a small conversion of the strongly immobilized signal to the weakly immobilized signal (Fung and Ostrowski, 1982). For a quantitative assessment of cross-linking effects on motions of spectrin, it is necessary to determine amounts of weakly and strongly immobilized components in each sample by spectral subtraction methods. We were able to remove the weakly immobilized component to give single-component spectra of strongly immobilized components for all four cross-linked samples (Fig. 7). From single-component (difference) spectra, we found that spectra of control (skeletal protein) samples

TABLE 1 7% Polyacrylamide gel electrophoresis results of control and cross-linked skeletal protein samples with and without dithiothreitol

	Without Dithiothreitol								With Dithiothreitol			
	Control		Diamide		Hydrogen Peroxide		Phenylhydrazine		Control		Glutaraldehyde	
	Int*	%	Int*	%	Int*	%	Int*	%	Int*	%	Int*	%
VHMWCs‡	–	0	–	59	–	1	–	13	–	0	–	53
HMWCs§	1637	29	1771	31	2841	50	2018	35	–	0	2053	46
1–2–4.1–5¶	4084	71	572	10	2842	49	2982	52	4445	100	45	1
Total‖	5721	100	2343	41	5683	99	5000	87	4445	100	2098	47

* Integrated intensity.

‡ VHMWCs (very high molecular weight complexes), defined as the proteins that did not enter the gel. The amount of VHMWCs (%) is estimated as follows: [Total intensity (control) − Total intensity (cross-linked)]/Total intensity (control).

§ HMWCs (high molecular weight complexes) defined as the proteins with molecular weight above that of band 1. The amount of HMWCs is determined from the integrated intensities of bands above the "37 line" (see Methods).

¶ Bands 1, 2, 4.1, and 5, defined as the proteins with molecular weight equal to or below that of band 1. The amount is determined from the integrated intensities of bands below the "37 line."

‖ Sum of intensities of HMWCs and 1–2–4.1–5.

consisted of 89.6 ± 0.4% (n = 11) strongly immobilized component and 10.4 ± 0.4% weakly immobilized components. These values correlate very well with literature values of 90% strongly immobilized component and 10% weakly immobilized component (Fung, 1983). For samples cross-linked at either SH or ϵ-NH_2 sites, the values were 86.3 ± 0.4% (n = 11) strongly immobilized component and 13.7 ± 0.4% weakly immobilized component for samples treated with 20-mM glutaraldehyde, 84.8 ± 0.2% (n = 4) strongly immobilized component and 15.2 ± 0.2% weakly immobilized component for samples treated with 1-mM diamide, 89.1 ± 0.2% (n = 4) strongly immobilized component and 10.9 ± 0.2%weakly immobilized component for samples treated with 0.5-mM hydrogen peroxide, and 85.3 ± 0.5%

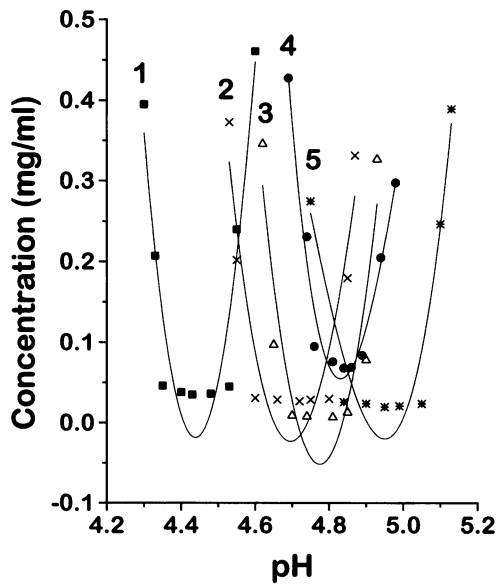

FIGURE 4 Plots of pH values and concentrations of skeletal protein samples. Third-order polynomial functions provided by the Origin software were used to group data points of each sample for ease of reading and had no theoretical significance. The pH value of the sample with minimum concentration was 4.44 for skeletal proteins (*curve 1*), 4.70 for the sample treated with phenylhydrazine (*curve 2*), 4.77 for the sample treated with glutaraldehyde (*curve 3*), 4.83 for the sample treated with hydrogen peroxide (*curve 4*), and 4.90 for the sample treated with diamide (*curve 5*).

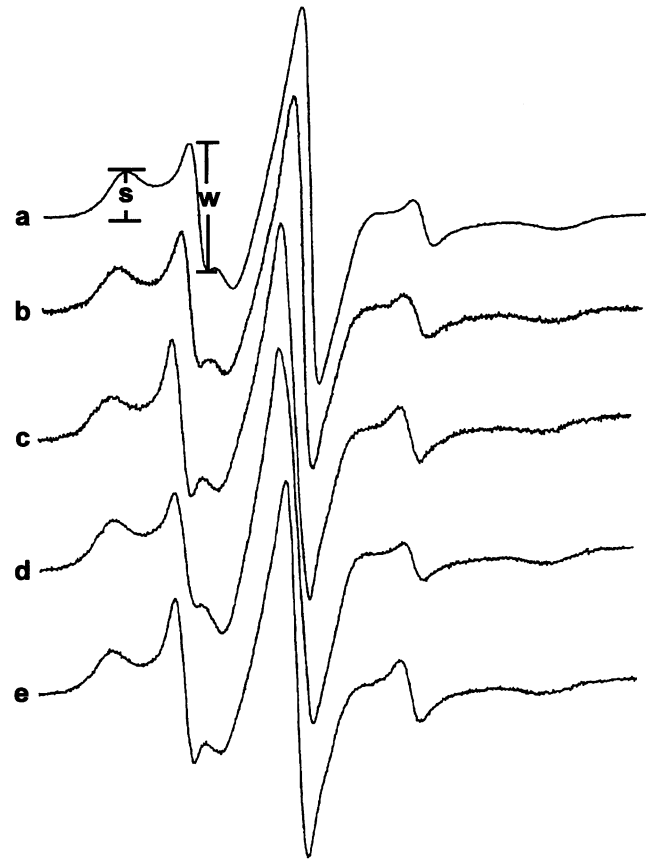

FIGURE 5 Conventional EPR spectra of (*a*) spin-labeled skeletal proteins and of skeletal proteins treated with (*b*) glutaraldehyde, (*c*) diamide, (*d*) hydrogen peroxide, and (*e*) phenylhydrazine. The signal amplitudes W and S are shown in spectrum *a*.

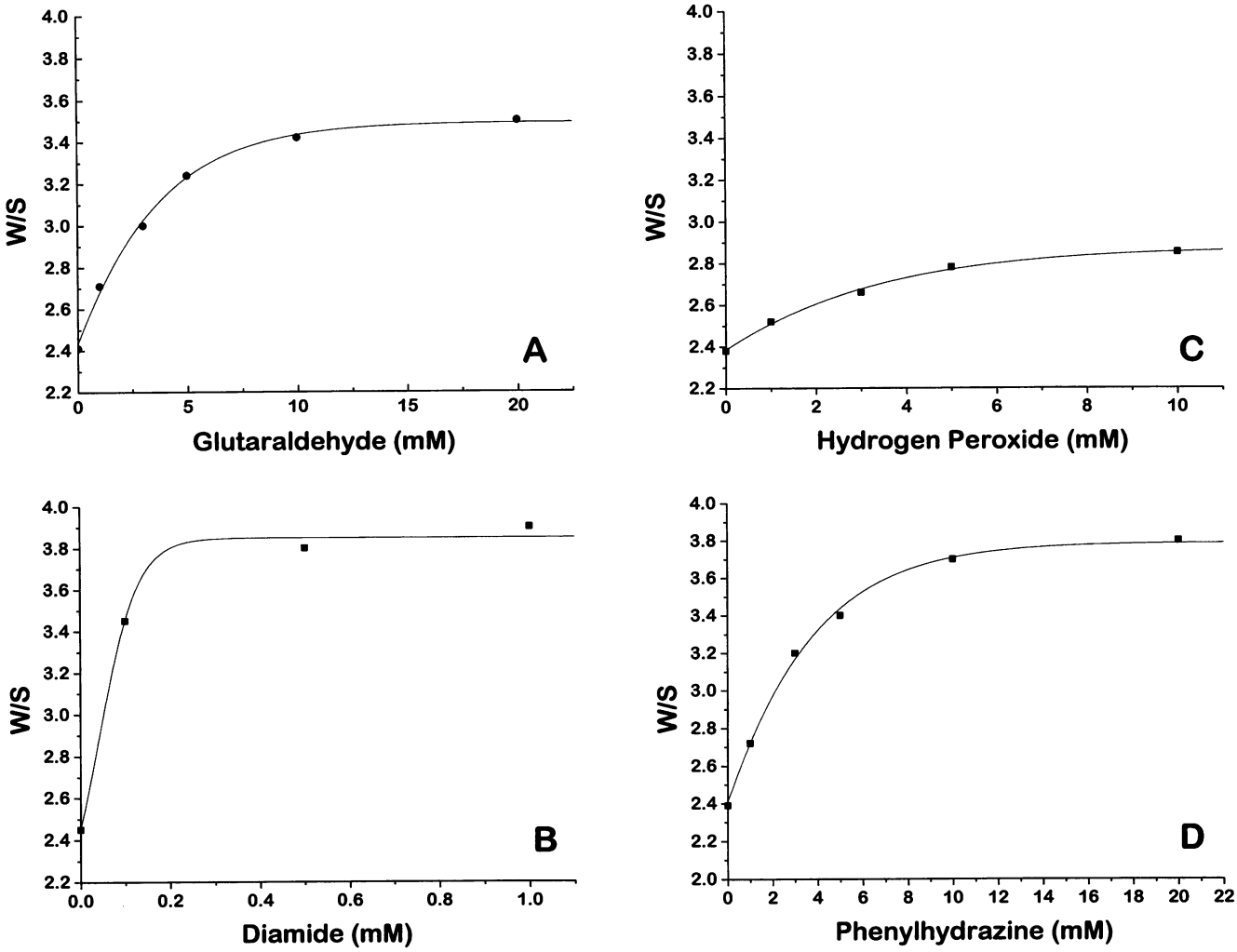

FIGURE 6 The *W/S* ratio values of spin-labeled skeletal protein samples treated with various amounts of cross-linking reagents: (*A*) glutaraldehyde at 22°C for 20 min, (*B*) diamide at 37°C for 60 min, (*C*) hydrogen peroxide at 37°C for 15 min, (*D*) phenylhydrazine at 37°C for 60 min.

(n = 3) strongly immobilized component and 14.7 ± 0.5% weakly immobilized component for samples treated with 20-mM phenylhydrazine. Thus, 0.5–5% of the strongly immobilized motional components were converted to weakly immobilized components in the four cross-linked samples.

As we were able to remove the weakly immobilized component in spectra of cross-linked samples with the weakly immobilized component spectrum (difference spectrum) of the control sample, the rotational correlation times of weakly immobilized components in all cross-linked samples were similar to those in skeletal protein samples, $\sim 10^{-9}$ s (Fung, 1981).

Segmental motions

Most of the segmental motions seen in spectrin in skeletal protein samples were in the range of 10^{-3}–10^{-7} s. Variation of these motions cannot be monitored by conventional EPR methods because of detection insensitivity but can be monitored by ST EPR methods (Fung and Johnson, 1983). Typical ST EPR spectra of samples with and without either glutaraldehyde or diamide (at saturating concentrations) at 20°C are shown in Fig. 8 *A* for pH 8 and in Fig. 8 *B* for pH 6. Again composite motional signals of weakly and strongly immobilized components were observed. The composite spectra of treated samples were similar to those of control samples under the same experimental conditions. Slight differences in spectral features mostly reflect different amounts of weakly immobilized component overlapping with the strongly immobilized component. Again, spectral subtraction allowed us to remove the weakly immobilized component to give spectra of strongly immobilized motions (Fig. 8 *C* and *D*). These difference spectra at either pH 8 or pH 6 were quite similar to each other, in L''/L, C'/C, and H''/H ratios (Table 2). The individual spectral amplitude ratios of L''/L for the three systems at pH 8 ranged from 0.51 to 0.77. These spectral amplitude ratios correspond to apparent rotational correlation times, $\tau(L''/L)$, of 3.0×10^{-6}–9.2×10^{-5} s, with experimental uncertainties ranging from 0 to 5.1×10^{-5} s (Table 3). Similar findings were observed for $\tau(C'/C)$, with values in the range of $1.5 \times$

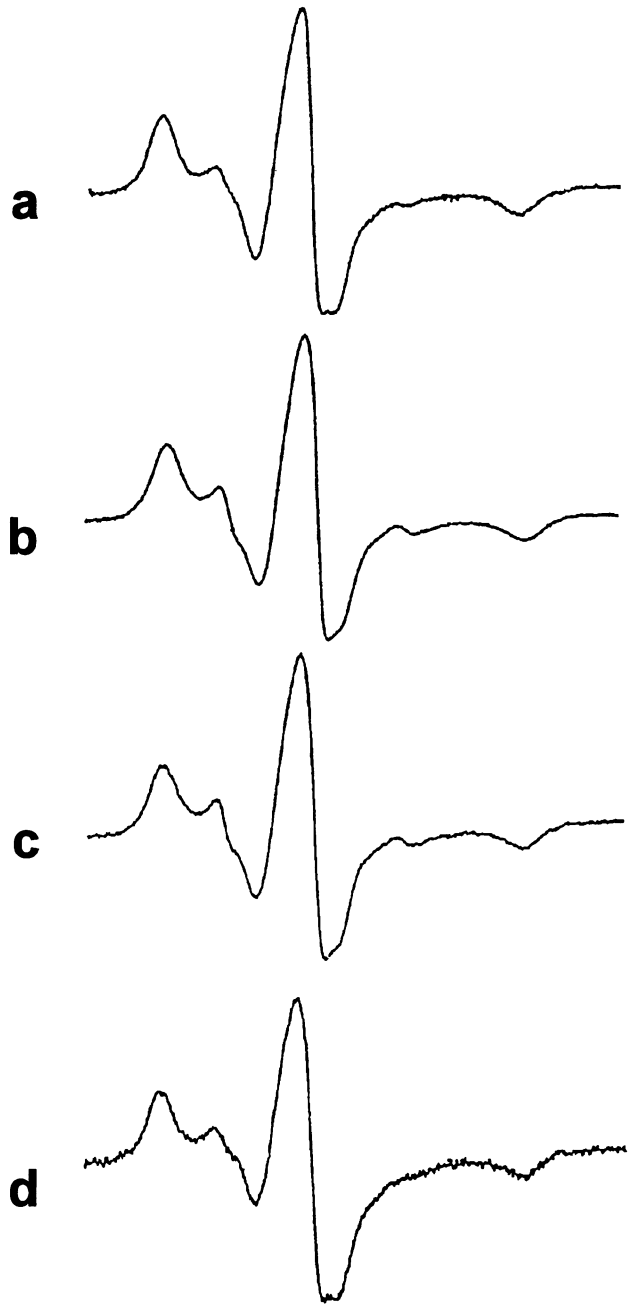

FIGURE 7 Conventional EPR difference spectra of samples treated with (*a*) glutaraldehyde, (*b*) diamide, (*c*) hydrogen peroxide, and (*d*) phenylhydrazine.

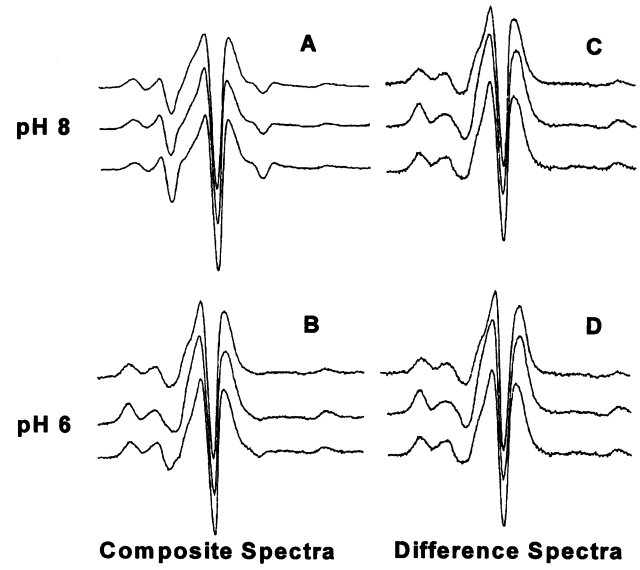

FIGURE 8 ST EPR composite spectra (*left column*) of spectrin (*top*), of samples treated with glutaraldehyde (*middle*), and of a sample treated with diamide (*bottom*) at pH 8 (*A*) and at pH 6 (*B*). ST EPR difference spectra (*right column*) of spectrin (*top*), of samples treated with glutaraldehyde (*middle*), and of a sample treated with diamide (*bottom*) at pH 8 (*C*) and at pH 6 (*D*).

10^{-6}–3.2×10^{-6} s and measurement uncertainties ranging from 0 to 0.8×10^{-6} s, and for $\tau(H''/H)$, with values in the range 3.5×10^{-6}–8.0×10^{-5} s and measurement uncertainties ranging from 0 to 0.8×10^{-6} s. τ values obtained from these three spectral regions were different, indicating that the segmental motions were anisotropic. Some motional components, such as $\tau(L''/L)$ values, of control and glutaraldehyde modified samples at pH 8 appeared to be quite different, whereas other components, such as $\tau(C'/C)$ at pH 8, appeared to be identical. We applied Student's *t*-tests to rotational correlation time values of skeleton and of skeleton with glutaraldehyde and also to values of skeleton and of skeleton with diamide. The results are shown in Table 4. These values show that most of the differences seen between values of skeleton and skeleton with glutaraldehyde or diamide are not statistically significant, except for $\tau(L''/L)$ of skeleton and skeleton with glutaraldehyde at pH 8, with a *t* of 42.2 and at pH 6, with a *t* of 8.3, and for $\tau(C'/C)$ of skeleton and of skeleton with glutaraldehyde at pH 8, with a *t* of -8.9. However, the results of these tests are questionable because only three experimental points were used (DF = 2). For a more rigorous analysis, we combined the full set of data by taking the ratio τ (modified)/τ (control), which eliminates the apparent differences between correlation times, as calculated from the *L*, *C*, and *H* parameters. For statistical analysis, we used the log[τ (modified)/τ (control)] values to obtain a symmetric distribution. In this second method of statistical analysis, if the τ values of control and modified samples are the same, the τ (modified)/τ (control) ratios will have a value of 1, and the log[τ (modified)/τ (control)] values in Table 5 will be 0. If the τ values of control and modified samples are different, the ratios will not be 1, and log[τ (modified)/τ (control)] values will not be 0. Thus, if the mean values of log[τ (modified)/τ (control)] are significantly different from 0, the τ values of control and modified samples will be statistically different. We found that for pH 8 the mean value was -0.04 and the SE was 0.106. The mean value for pH 6 systems was -0.06, and the SE was 0.110. Both mean values suggest that the rotational correlation times of control and modified samples are equivalent within the experimental uncertainties. The agreement between the values of the SE obtained at pH 8 and those at pH 6 provides some verification for

TABLE 2 ST EPR spectral parameters of spin-labeled spectrin in skeletal protein samples with and without cross-linking reagents

	L''/L		C'/C		H''/H	
	Composite	Difference	Composite	Difference	Composite	Difference
pH 8						
Skeleton	0.94 ± 0.03	0.72 ± 0.00	−1.93 ± 0.12	−0.96 ± 0.07	0.41 ± 0.03	0.37 ± 0.01
Skeleton + glutaraldehyde	1.52 ± 0.04	0.57 ± 0.01	−1.83 ± 0.20	−0.79 ± 0.03	0.61 ± 0.04	0.35 ± 0.01
Skeleton + diamide	1.73 ± 0.06	0.63 ± 0.12	−1.75 ± 0.19	−0.93 ± 0.09	0.48 ± 0.04	0.45 ± 0.02
pH 6						
Skeleton	0.76 ± 0.01	0.74 ± 0.02	−1.13 ± 0.01	−0.91 ± 0.02	0.31 ± 0.01	0.37 ± 0.00
Skeleton + glutaraldehyde	0.66 ± 0.01	0.58 ± 0.01	−0.90 ± 0.05	−0.75 ± 0.04	0.46 ± 0.01	0.34 ± 0.02
Skeleton + diamide	0.90 ± 0.04	0.65 ± 0.12	−1.15 ± 0.14	−0.95 ± 0.00	0.47 ± 0.02	0.42 ± 0.03

The values of the samples were mean values of at least three experiments. L''/L, C'/C, and H''/H are amplitude ratios of ST EPR spectra in the low-field, central-field, and high-field regions, respectively.

the approach. Student's t-tests of the null hypothesis confirm that there is no significant difference between the modified and control populations (t = -0.532 and prob = 0.617 for pH 8 and t = -0.328 and prob = 0.757 for pH 6, each with DF = 5. If we combined pH 8 and pH 6 data, we obtained a t of -0.639 and a prob of 0.536, with DF = 11). This approach for analyzing τ values should be more reliable for evaluating whether the motional components of control and modified samples were similar or different from each other because it permits a single analysis of the full population. This method of taking the ratio of τ (modified)/τ (control) and then applying a logarithm to the ratio gives equal dispersion for ratios greater or less than 1 and avoids uncertainties from individual measurements. We believe that it is a more rigorous evaluation for comparing the full set of data under different experimental conditions for control versus modified samples than Student's t-test comparing three experimental points under one experimental condition. The τ values that appeared to be significantly different by Student's t-test criteria were probably artifacts of the small number of data points used for each measurement. The standard errors of the mean of 0.106–0.110 (SE in Table 5) in values of log[τ (modified)/τ (control)] indicate that the ratio, [τ (modified)/τ (control)], is uncertain within a factor of ~1.3; i.e., that within a factor of 1.3 oxidation does not affect the segmental motions as measured by ST EPR correlation times.

Generally speaking, we were not able to detect significant decreases in the apparent rotational correlation times by ST EPR at 9 GHz. Inasmuch as all the spectral features were quite similar, it was not necessary to use 35 GHz to resolve further the different types of strongly immobilized segmental motion as reported earlier (Fung and Johnson, 1983). All three principal motional components, with correlation times of $\leq 10^{-9}$, 10^{-7}–10^{-6}, and $\sim 10^{-3}$ s, were not restricted by oxidation. Our data also show that the motions detected by ST EPR appear to be independent of pH over the range 6–8, in good agreement with previously published data (Fung and Johnson, 1983).

TABLE 3 Rotational correlation times obtained from difference ST EPR spectra of spin-labeled spectrin in skeletal protein samples with and without cross-linking reagents (Table 2)

	$\tau(L''/L)$ (10^{-5} s)	$\tau(C'/C)$ (10^{-6} s)	$\tau(H''/H)$ (10^{-5} s)
pH 8			
Skeleton	7.5 ± 0.0	1.5 ± 0.4	4.5 ± 0.5
Skeleton + glutaraldehyde	2.6 ± 0.2	2.5 ± 0.6	3.8 ± 0.3
Skeleton + diamide	5.3 ± 4.2	1.5 ± 0.5	8.0 ± 1.0
pH 6			
Skeleton	9.2 ± 1.8	1.7 ± 0.1	4.5 ± 0.0
Skeleton + glutaraldehyde	3.0 ± 0.5	3.2 ± 0.8	3.5 ± 0.5
Skeleton + diamide	6.3 ± 5.1	1.6 ± 0.0	6.7 ± 1.3

DISCUSSION

Current models describing red cell elasticity emphasize the freedom of motion or movement of molecules relative to one another in the membrane skeleton, and thus it is generally agreed that rigidification through cross-linking of membrane proteins decreases elasticity (Mohandas, 1992; Nash and Gratzer, 1993; Mohandas and Evans, 1994). However, it was found that dissociation of native spectrin tetramers to dimers resulted in increases in rigidity (Chabanel et al., 1989). Thus the relationship between spectrin self-interactions and membrane rigidity is quite complex (Nash and Gratzer, 1993). Spectrin has been considered an entropic spring, where its flexibility is derived from configurational entropy similar to the elasticity of rubber (Elgsaeter et al., 1986). However, studies have also shown that the elastic deformability of spectrin and of the erythrocyte arises from reversible dissociations of weak but specific intramolecular contacts rather than from configurational entropy (Vertessy and Steck, 1989). Recent NMR studies indicate that the thermal flexing of spectrin is not due solely to configurational entropy but includes enthalpic components also (Begg et al., 1994). Generally speaking, biological macromolecules can be classified into two general types, rigid and flexible (Garcia de la Torre, 1994). Without conformational variability, the macromolecule can be re-

TABLE 4 Paired Student's *t*-tests on rotational correlation times values obtained in Table 3 with two degrees of freedom

	Mean Difference $\tau(L''/L)/\tau(C'/C)/\tau(H''/H)$	SE Difference $\tau(L''/L)/\tau(C'/C)/\tau(H''/H)$	t $\tau(L''/L)/\tau(C'/C)/\tau(H''/H)$	Probability $\tau(L''/L)/\tau(C'/C)/\tau(H''/H)$
pH 8				
Skeleton/skeleton + glutaraldehyde	4.9/−1.1/0.7	0.2/0.2/0.8	42.4/−8.9/1.7	0.0/0.0/0.2
Skeleton/skeleton + diamide	2.2/−0.1/−3.5	4.2/0.1/1.5	0.9/−2.0/−4.0	0.5/0.2/0.1
pH 6				
Skeleton/skeleton + glutaraldehyde	6.2/−1.4/1.0	1.3/0.7/0.5	8.3/−3.5/3.5	0.0/0.1/0.1
Skeleton/skeleton + diamide	2.9/0.1/−2.2	3.4/0.1/1.3	1.5/4.0/−3.0	0.3/0.1/0.1

garded as a rigid particle. On the other hand, the flexibility of biological macromolecules may play an essential role in their physiological functions. In the rigid case that role is determined by the peculiarities of the shape, whereas in the flexible ones function is usually linked to flexibility. Spectrin molecules in membrane skeleton are highly flexible with various detectable segmental motions under physiological conditions, as discussed earlier. Substantial immobilization of spectrin in the membrane skeleton was observed in samples at pH 4.5 (Fung and Johnson, 1983). Increase in mobility was observed at high pH (Fung and Ostrowski, 1982) and high temperature (Begg et al., 1994).

In this study, though we detected some motional variations in samples modified with oxidants, we clearly demonstrated that the flexibility of spectrin molecules was not decreased. The protein modifications resulted in the formation of VHMWCs and HMWCs. These complexes exhibited altered molecular properties, such as different surface charge properties, to give different pI values. However, their segmental motions remained very similar to those observed in samples without cross-linking reagents. Though it was initially surprising that the segmental motions in cross-linked skeletal protein complexes were not restricted when compared with those in nonmodified forms, this phenomenon may be explained by the fact that the skeletal protein network is composed of relatively rigid subunits linked by joints that are more-or-less flexible. Elastic materials are typically networks of cross-linked filaments (Mark, 1981). The native spectrin–actin–protein 4.1 samples in SDS gel exhibit clear and distinct monomeric protein bands with no VHMWC or HMWC bands, whereas the oxidized samples exhibit dramatic alterations in band patterns. However, this SDS gel picture may be misleading. The native membrane skeleton consists of numerous noncovalent molecular interactions, with association of the various proteins to form a skeletal network (Steck, 1989). Spectrin in this skeletal network exhibits characteristic segmental motions, and these motions appear to be the same for spectrin in solution and in membranes, as previously reported by us (Fung and Johnson, 1983) and by others (Learmonth et al., 1989), suggesting that the attachment of skeletal proteins to other proteins and/or lipid bilayers at their anchoring positions did not alter the segmental motions of spectrin. Although it is often assumed that spectrin flexibility is the molecular origin of the unique deformability and elasticity of erythrocytes, this study shows that increased cellular rigidity and membrane stiffness in oxidized erythrocytes cannot be attributed to the restriction of spectrin segmental motions, because these motions are largely unaffected when cross-linked by glutaraldehyde or oxidized by diamide or hydrogen peroxide. These findings have clear implication not only for understanding spectrin segmental motions but also for understanding, in general, oxidation induced cell rigidity.

Our studies are focused on the segmental motions of spectrin in solution when skeletal proteins are cross-linked, similar to diamide oxidation processes in erythrocytes in which only skeletal proteins are cross-linked (Haest et al., 1977). Protein oxidation in intact erythrocytes is often complicated by the presence of hemoglobin and lipid molecules. For example, cross-linking of membrane proteins by disulfide exchange with precipitated hemoglobin was found when phenylhydrazine was added to intact erythrocytes, and the decreased cell deformability is attributed to the cross-linking of spectrin with oxidized hemoglobin (Vilsen and Nielsen, 1984; Chakrabarti et al., 1995). It will be interesting to study segmental motions and/or translational motions of spectrin in samples with spectrin cross-linked to oxidized hemoglobin.

TABLE 5 Comparison of rotational correlation time measurements of modified and control samples

	log [τ (modified)/τ (control)]					
	pH 8			pH 6		
Modifier	L''/L	C'/C	H''/H	L''/L	C'/C	H''/H
Glutaraldehyde	−0.46	+0.22	−0.07	−0.49	+0.27	−0.11
Diamide	−0.15	0.0	+0.25	−0.16	−0.03	+0.17
Mean ± SE	−0.04 ± 0.106 (n = 6)			−0.06 ± 0.110 (n = 6)		
t*	−0.328			−0.532		
DF	5			5		
Prob.	0.757			0.617		

* Student's t-test with null hypothesis. t = −0.639, DF = 11, Prob. = 0.536 when pH 8 and pH 6 data were combined.

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