Dynamics and Interactions of the Anion Channel in Intact Human Erythrocytes: An Electron Paramagnetic Resonance Spectroscopic Study Employing a New Membrane-Impermeant Bifunctional Spin-Label†

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Received November 18, 1985; Revised Manuscript Received January 29, 1986

ABSTRACT: We have developed a new membrane-impermeant, bifunctional spin-labeling reagent, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate (BSSDP), and employed it in an electron paramagnetic resonance (EPR) study of the rotational diffusion of the anion-exchange channel (band 3) in *intact* human erythrocytes. BSSDP reacts in a covalent manner and with high specificity with the extracytoplasmic domain of band 3, forming a complex in which the spin-label is immobilized on the protein. The linear EPR spectrum of BSSDP-labeled intact erythrocytes is characteristic of a highly immobilized, spatially isolated nitroxide probe. The saturation-transfer EPR spectrum of the same sample indicates that the anion channel in intact erythrocytes exhibits rotational dynamics in the 0.1–1 ms correlation time range at 20 °C. Rotational dynamics in this motional domain are consistent with a strong interaction of the anion-exchange channel with the erythrocyte cytoskeleton. The saturation-transfer EPR spectrum of ghosts prepared from BSSDP-labeled erythrocytes indicates a significant increase in rotational mobility of the anion channel, suggesting a significant disruption on lysis of interactions between the anion channel and the cytoskeleton.

The interaction of the anion-exchange channel (band 3) of the human erythrocyte membrane with the cytoskeletal matrix has been the focus of considerable attention during the past several years. In an early experiment, Peters et al. (1974) attempted to measure the lateral mobility of labeled membrane proteins, including band 3, by fluorescence recovery after photobleaching half of an individual erythrocyte ghost prepared from a fluorescein-labeled cell. On the time scale of their measurements (20 min at room temperature) there was no detectable recovery. The authors concluded that 3×10^{-12} cm^2/s was an upper limit diffusion coefficient for labeled membrane proteins under their conditions.

Fowler and Branton (1977) fused erythrocytes that had been labeled with fluorescein isothiocyanate, principally on band 3 and glycophorin (PAS-I), with unlabeled cells and measured the redistribution of label by scoring the percentage of uniformly labeled cells in a microscopic field with time. The authors found that redistribution was strongly temperature dependent and was also dependent on whether the erythrocytes were fresh or not. For fused cells prepared from fresh erythrocytes, they calculated minimum diffusion constants of 6×10^{-12} cm^2/s at room temperature and 4×10^{-11} cm^2/s at 37 °C. This mobility is lower than would be expected for freely diffusing band 3 and glycophorin in a lipid bilayer matrix (Saffman & Delbruck, 1975). This observed restriction in translational mobility is consistent with the interaction of these proteins with the cytoskeletal matrix.

Cherry and co-workers (Cherry et al., 1976; Nigg & Cherry, 1979a,b; Nigg et al., 1980) have measured the rotational mobility of band 3 in ghost membranes prepared from cells labeled with the triplet probe eosin. These studies have employed a method in which a population of probe molecules is excited by a pulse of plane-polarized light and the decay of dichroism of absorption of the resulting triplets is measured (Cherry, 1978). The data have been interpreted as arising from two populations of band 3, one with a rotational correlation time of approximately 0.2 ms at 37 °C and the other of 2–5 ms at that temperature (Cherry & Nigg, 1980).

The molecular basis for the observed restriction in translational and rotational diffusion of band 3 has been elucidated, at least in part, by the discovery of a protein, termed ankyrin (band 2.1, and the sequence-related bands 2.2–2.6), which binds band 3 and spectrin (Bennett, 1978; Luna et al., 1979; Bennett & Stenbuck, 1979a,b, 1980a,b; Tyler et al., 1979; Yu & Goodman, 1979). It is proposed that a population of band 3 molecules is bound to ankyrin which, in turn, is bound to spectrin in the cytoskeletal network, giving rise to a low-mobility population of anion channels.

In the studies cited above on the dynamics of band 3 in the membrane, measurements were made on red cell ghosts or on cells disrupted by fusion. It is the goal of the studies presented here to measure the rotational dynamics of band 3 in the membranes of *intact* erythrocytes and thereby to observe changes in mobility resulting from disruption of the cells. To this end, we have prepared a new membrane-impermeant, reactively bifunctional spin-label that has a very high affinity for the anion channel. Cells labeled with this reagent have been monitored by linear and saturation-transfer EPR[1] tech-

† This work was supported in part by National Institutes of Health Grants HL30372, HL34737, and AM31880. A.H.B. is a recipient of a Searle Scholar Award/Chicago Community Trust.

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[1] Abbreviations: BSSDP, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate (throughout this paper, the use of doxylpimelate, doxylstearate, etc. implies a spiro compound, with the locant preceding doxyl referring to the carbon on the acid group that is common to the doxyl ring); doxyl, 4,4-dimethyloxazolidine-*N*-oxyl; PAS, periodic acid–Schiff; EPR, electron paramagnetic resonance; V_1, first harmonic, in-phase absorption EPR signal; ST-EPR, saturation-transfer EPR; V_2', second harmonic, out-of-phase absorption EPR signal; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BS^3, bis(sulfo-*N*-succinimidyl) suberate; τ, correlation time for isotropic rotational diffusion.

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niques to measure the interactions and mobility of band 3 in these intact cells. A preliminary account of some of this work has been published in abstract form (Beth et al., 1985).

Experimental Procedures

Synthesis of Bis(sulfo-N-succinimidyl) Doxyl-2-spiro-4'-pimelate. The synthesis of 4-doxylpimelate from diethyl 4-ketopimelate was accomplished by modifications of procedures for conversion of keto groups to doxyl groups (Keana et al., 1967; Hubbell & McConnell, 1971). A 500-mL three-neck flask was equipped for anhydrous reflux with a Dean–Stark water trap between the flask and condenser. The flask was charged with 100 mL of dry toluene, 11.5 g (50 mmol) of diethyl 4-ketopimelate (Aldrich Chemical Co.), 100 mg (0.5 mmol) of *p*-toluenesulfonic acid monohydrate (Aldrich Chemical Co.), and 45 g (0.5 mol) of 2-amino-2-methyl-1-propanol (Aldrich Chemical Co.) and was purged with nitrogen. The reaction mixture was gently refluxed for 6 days, during which time 5 mL of water plus amino alcohol collected in the trap. After the mixture was cooled to room temperature, toluene was removed under reduced pressure on a rotary evaporator, and the remaining orange residue was transferred to a round-bottom flask equipped for vacuum distillation. The unreacted amino alcohol was removed at 40 °C (0.01 mmHg), leaving behind 17 g of a thick orange oil. Residual amino alcohol was removed by connecting the flask to a vacuum line (0.01 mmHg) and immersing the flask in a regulated 50 °C water bath overnight. The resulting orange semisolid was dissolved in 200 mL of dry methanol and stored over anhydrous sodium carbonate for 2 days at room temperature.

Sodium carbonate was removed by filtration and methanol removed under reduced pressure on a rotary evaporator. The resulting orange semisolid (15 g) was dissolved in 100 mL of diethyl ether, and the sample was filtered into a 250-mL Erlenmeyer flask. A solution of 14 g of *m*-chloroperoxybenzoic acid (Aldrich Chemical Co.) in 50 mL of diethyl ether at 2 °C was added dropwise to the stirred solution of crude amine from above at the same temperature. The oxidation reaction was allowed to proceed for 36 h in the cold, during which time the reaction mixture became increasingly yellow in color. The reaction was terminated by removing the ether under reduced pressure, leaving behind a yellow solid. The desired product was solubilized from the solid, and the majority of the oxidizing agent was left behind by extracting the solid 3 times with 5-mL portions of distilled water. The water layer was yellow and gave a strong EPR signal characteristic of a doxyl nitroxide. The pH of the water layer was adjusted to 3.5 with 1 N HCl and then was extracted 3 times with 25-mL portions of ethyl acetate. The ethyl acetate layers were combined over 5 g of anhydrous sodium sulfate and dried overnight.

Sodium sulfate was removed by filtration, and ethyl acetate was evaporated on a rotary evaporator to yield 2 g of a yellow oil. The oil was dissolved in 2 mL of 10 mM ammonium formate, pH 3.5/methanol (70/30 v/v), and the sample was filtered through a 5-μm nylon filter (Pall). The diester of 4-doxylpimelate was purified by preparative reverse-phase HPLC on a Beckman 344 gradient HPLC system fitted with a 2.2 by 25 cm Partisil ODS-3 column (Whatman) and a 1-mL sample loop, using a mobile phase of 10 mM ammonium formate, pH 3.5/methanol (70/30 v/v) eluted at 5.6 mL/min. The major peak in the elution profile detected at 254 nm was collected from two successive runs, combined, and lyophilized to yield 0.6 g of a yellow semisolid.

The diester was saponified by dissolving the lyophilized sample in 20 mL of methanol, followed by adding dropwise 5 mL of 20% aqueous sodium hydroxide. After the solution was stirred for 48 h, the methanol was removed under reduced pressure, and the pH of the aqueous residue was adjusted to 3.5 with HCl. The diacid produced was extracted into three 25-mL portions of ethyl acetate, and the combined extracts were dried over sodium sulfate. Following removal of ethyl acetate, the sample was dissolved for preparative HPLC in 5 mL of a mobile phase of 10 mM ammonium formate, pH 3.5/methanol (85/15 v/v), and the sample was applied in successive runs to the column described above and was eluted at 5.6 mL/min. The major peak detected at 213 nm was collected and combined from each of the runs. Solvent and buffer were removed by lyophilization to yield 0.3 g of a yellow solid. The solid was dissolved in 5 mL of distilled water, the pH adjusted to 3.0 with 1 N HCl, and the product extracted into ethyl acetate. The ethyl acetate was removed under reduced pressure and the sample dried in vacuo over phosphorus pentoxide to yield 0.3 g of product. Fast atom bombardment mass spectral analysis indicated a molecular ion at 260 mass units characteristic of formation of the hydroxylamine of 4-doxylpimelic acid less H^+. Standard runs on 5-doxylstearic acid (Aldrich Chemical Co.) also gave the hydroxylamine of the parent nitroxide as the molecular ion under these conditions.

Bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4'-pimelate (BSSDP)[1] was synthesized by the general method for the synthesis of bis(sulfosuccinimidyl) esters of dicarboxylic acids (Staros, 1982). Briefly, 0.138 g (0.53 mmol) of 4-doxylpimelate, 0.230 g (1.06 mmol) of *N*-hydroxysulfosuccinimide (Staros, 1982), and 0.250 g (1.21 mmol) of dicyclohexylcarbodiimide (Aldrich Chemical Co.) were taken up in 0.27 mL of dry *N*,*N*-dimethylformamide. The reaction mixtue was stirred at room temperature overnight and then at 3 °C for 4 h, after which time the dicyclohexylurea was removed by filtration and washed with a small volume of *N*,*N*-dimethylformamide. The product was precipitated from the filtrate by addition of 200 mL of ethyl acetate, and the product was collected by filtration and dried in vacuo over phosphorus pentoxide. The final product was a pale yellow solid, which was subjected to negative ion fast atom bombardment mass spectral analysis.

The expected mass for the hydroxylamine of Na_2BSSDP ($C_{19}H_{23}O_{16}N_3S_2Na_2$) is 659 mass units (British standard mass units, which are used to calibrate our mass spectrometer). The loss of sodium from the hydroxylamine results in a negative ion with mass 636. This is the major peak that we see in the negative ion mode fast atom bombardment mass spectrum of BSSDP and is consistent with the fragmentation patterns of other sulfosuccinimidyl esters that have been examined by this technique (Anjaneyulu & Staros, 1986). Also detected are fragments at 260 mass units, corresponding to the hydroxylamine of 4-doxylpimelic acid less H^+, 216 mass units, corresponding to sodium *N*-hydroxysulfosuccinimide less H^+, and 194 mass units, corresponding to *N*-hydroxysulfosuccinimide (i.e., loss of Na^+).

In addition to mass spectral analysis, we have also followed the hydrolysis of BSSDP by HPLC (Staros, 1982). These studies indicated that the only final hydrolysis products that could be detected were 4-doxylpimelate and *N*-hydroxysulfosuccinimide in 1:2 molar ratio based on separately chromatographed standards.

We have determined spins/mol of BSSDP from integration of EPR spectra using twice recrystallized 5-doxylstearic acid and 16-doxylstearic acid (Aldrich Chemical Co.) as standards. Two separate weighings of BSSDP and each of the standards resulted in estimates ranging from 0.9 to 1.0 spin/mol in our BSSDP preparation.

EPR Measurements. EPR measurements were made with a Varian E-112 spectrometer equipped with an E-238 high-volume aqueous cavity. Samples were contained in a standard WG-813 flat cell (Wilmad, Buena, NJ). The sample temperature was continuously monitored with a digital thermometer (Baily BAT12-R) by placing a temperature probe into the flat cell in contact with the sample. The sample temperature was regulated during measurements with an E-257 temperature controller by passing precooled air into the cavity through the radiation slots in the front. This resulted in a 1-deg temperature gradient over the active dimensions of the sample. Linear EPR signals (V_1) were recorded at 100-kHz field modulation of 1-G amplitude (peak–peak) with a microwave observer power of 10 mW. ST-EPR signals (V_2') were recorded at 50-kHz field modulation (100-kHz detection) of 5-G amplitude with a microwave observer power of 100 mW that corresponded to 0.2 G in the rotating frame [calibrated; Beth et al. (1983)]. The out-of-phase setting of the phase-sensitive detector was determined by the self-null method (Thomas et al., 1976) at a microwave power level of 0.5 mW. Signals were recorded digitally by an on-line PDP 11/23 microcomputer, which also drove the magnetic field sweep. Spectral subtraction and integration routines (trapezoidal rule) developed for this system were employed for data analysis.

Labeling of Intact Erythrocytes with BSSDP. Erythrocytes were prepared from heparinized whole blood freshly drawn from normal adult donors. All manipulations were at 0–3 °C unless otherwise noted. After separation from the plasma and buffy coat, erythrocytes were washed twice in phosphate-buffered saline (0.15 M NaCl, 14 mM sodium phosphate), pH 7.4, and then once in 106 mM sodium phosphate, pH 7.4. The resulting cells were resuspended to 50% hematocrit in the 106 mM sodium phosphate buffer. Aliquots of these cells were treated at room temperature with various concentrations of BSSDP added from a freshly prepared 10 mM stock in the same buffer. After a 30-min incubation at room temperature with gentle agitation, the reaction was quenched by addition of at least 3 volumes of 0.14 M NaCl, 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and 10 mM ethanolamine, pH 7.4, followed by centrifugation. The supernatant was retained to monitor lysis. Samples to be analyzed by EPR spectroscopy were then washed in 40 volumes of 0.15 M NaCl and 5 mM sodium phosphate, pH 7.4, containing 1% (w/v) bovine serum albumin (fraction V, Sigma), and finally twice in the same buffer without bovine serum albumin. The number of cells per milliliter in the final suspension was quantitated by counting a diluted aliquot in a Coulter counter (Model ZBI) with coincidence correction.

For spectral measurements on ghosts, erythrocytes labeled with BSSDP were lysed and washed free of hemoglobin and other cytosolic components as previously described (Staros & Richards, 1974) except that 100 μL of a 1 mg/mL stock of freshly prepared ascorbate oxidase (Sigma) was added to the hemolyzing medium to minimize chemical reduction of the spin-label. Ghosts were washed repeatedly and resuspended in a buffer of 15 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1.7 mM tris(hydroxymethyl)-aminomethane, pH 7.4, or in a buffer of 5 mM sodium phosphate, pH 7.4.

Proteins Labeled by BSSDP in Intact Erythrocytes. Ghosts prepared as above were pelleted by centrifugation, and the resulting pellet (0.95 mL) was solubilized by addition of 0.05 mL of 20% SDS. The resulting clear solution was spun for 20 min at 20000*g* and the small insoluble pellet removed. The supernatant (1 mL) was applied to a 1.0 × 60 cm TSK-3000-SW column equilibrated in 100 mM sodium phosphate buffer, pH 7.0, containing 0.3% SDS, and the column was eluted with the same buffer at 0.8 mL/min with a Beckman 112 HPLC pump. Protein elution was monitored at 280 nm with a UV detector. The amount of spin-label in each fraction from the column was determined by double integration of the recorded EPR signal. The protein composition in each fraction was determined by SDS–PAGE essentially as described by Fairbanks et al. (1971).

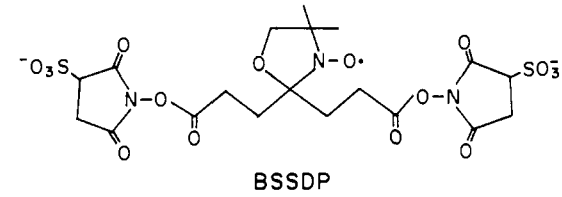

FIGURE 1: Chemical structure of BSSDP.

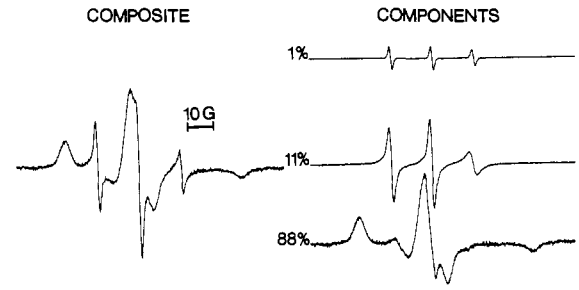

FIGURE 2: Deconvolution of the EPR spectrum from BSSDP-labeled erythrocytes. The composite EPR spectrum (left) was recorded on intact erythrocytes labeled with 50 μM BSSDP as described under Experimental Procedures. The spectrum consists of signals from BSSDP in three different motional environments. The three spectra on the right were obtained by sequential digital subtractions of EPR spectra obtained from (1) freely tumbling BSSDP in the same buffer (upper) and (2) partially immobilized BSSDP bound to membrane lipids (middle) to yield the residual slow-motion signal from BSSDP bound to membrane proteins (lower). The three component signals are displayed at the amplitude they contribute to the composite spectrum. Double integration of each component spectrum indicated that 1% of BSSDP was in the unassociated, freely tumbling environment, 11% was bound to membrane lipids, giving rise to the partially immobilized signal, and 88% was bound to membrane proteins, giving rise to the slow-motion signal. The composite spectrum was recorded at 20 °C in isotonic phosphate-buffered saline, pH 7.4.

RESULTS

Properties of Intact Erythrocytes Labeled with BSSDP. The bifunctional reagent BSSDP (Figure 1) binds rapidly to the exofacial surface of intact erythrocytes to yield covalently modified band 3 as the major product. The EPR spectrum of labeled erythrocytes (Figure 2, left) indicates that the majority of the label (>85%) is immobilized by covalent binding to membrane proteins (Figure 2, lower right). A partially immobilized component is also observed (Figure 2, middle right) that can be recovered from the organic phase of a 1/1 chloroform/methanol extract of ghosts prepared from BSSDP-labeled cells (Figure 3, lower), suggesting that this component arises from BSSDP-labeled lipids. This component comprised from 10% to 15% of the total integrated signal intensity when intact erythrocytes were labeled with BSSDP at concentrations of 50 μM or less. At concentrations of reagent from 50 to 150 μM there was a progressive increase in the partially immobilized component relative to the immobilized signal. The fast-motion, unbound signal (Figure 2, upper right) is from a small amount of label (1–2%) that could be removed by repeated washing of labeled cells. The three components observed are shown separately (Figure 2,

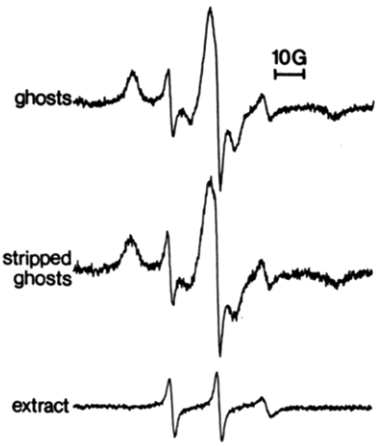

FIGURE 3: EPR spectra of erythrocyte ghost membranes, stripped membranes, and membrane lipids. Erythrocytes labeled with 50 μM BSSDP (Figure 2) were hemolyzed by dropwise addition to hypotonic buffer as described under Experimental Procedures. Hemoglobin and other cytosolic proteins were removed by repeated washing to yield white ghost membranes. The EPR spectrum of these ghost membrane suspended in the wash buffer (upper) retained 95% of the slow and intermediate motional components that were present in labeled erythrocytes. Integrated signal intensities were corrected for differences in cell and ghost counts. The small-amplitude fast-motion component observed in measurements on intact erythrocytes (Figure 2, upper right) was removed by preparation of ghosts. Removal of peripheral membrane skeletal proteins by extraction with 0.1 N NaOH resulted in recovery of stripped membranes, which retained the slow and intermediate motional components (middle). Ghost membranes prepared from labeled intact cells were extracted with a 1/1 mixture of chloroform/methanol to extract membrane lipids. Following removal of solvent and resuspension of the lipids by sonication, the intermediate-motion signal (lower) was observed. All spectra were recorded at 20 °C.

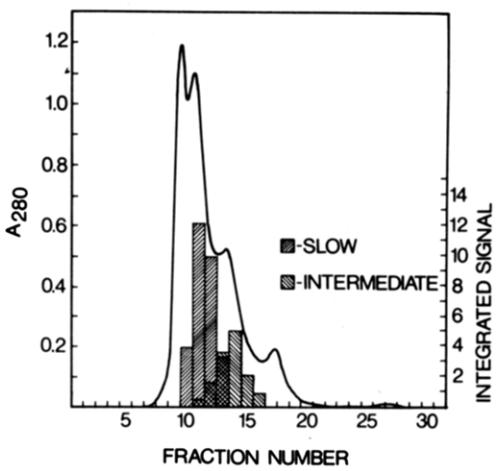

FIGURE 4: Gel permeation HPLC separation of erythrocyte membrane proteins. Ghost membranes prepared from erythrocytes labeled with 50 μM BSSDP were solubilized in 1% SDS. The resulting solution was applied to a TSK-3000-SW gel permeation column, and the proteins were eluted as described under Experimental Procedures. The solid tracing is the elution profile detected at 280 nm. Positions where fractions were collected from the column are shown on the abscissa. EPR spectra recorded from each fraction were analyzed for concentrations of BSSDP giving rise to slow- and intermediate-motion signals. The slow-motion signal was partially resolved from the intermediate-motion signal and comigrated with band 3 protein (Figure 5). No EPR signal was detectable in the column fractions that were rich in bands 1, 2, and 2.1 but contained no band 3. Recovery of signal was greater than 90% of that applied to the column in separate labeling experiments.

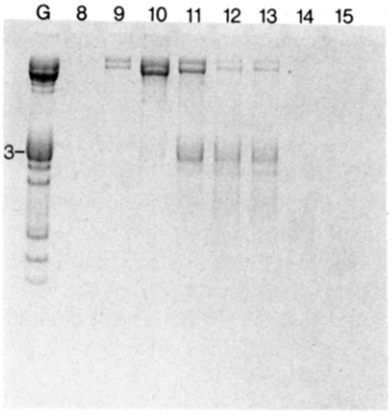

FIGURE 5: SDS–PAGE of column fractions. The protein composition of column fractions that contained detectable spin-label signal from the TSK column was determined by separating membrane proteins on SDS–PAGE. Protein bands were visualized by staining with Coomassie blue. A constant volume of sample from each fraction was applied to the gel so that staining intensities can be correlated directly with relative abundance of proteins. The left lane is from labeled ghost membranes prior to HPLC separation of proteins.

right) at the amplitude that they contribute to the composite (Figure 2, left).

The majority of the labels giving rise to the immobilized component in Figure 2, lower right, were covalently bound to band 3 protein. This specificity is demonstrated by the protein separations shown in Figures 3–5. Hypotonic lysis of labeled erythrocytes followed by removal of cytosolic proteins by repeated washing resulted in full retention of the immobilized component (Figure 2, lower right) and the partially immobilized component (Figure 2, middle right) in the ghost membranes (Figure 3, upper). Likewise, extraction of peripheral proteins including membrane skeletal proteins with 0.1 N NaOH, a procedure that produces membranes which retain band 3 and the integral PAS-staining proteins (Steck & Yu, 1973), resulted in retention of the immobilized and partially immobilized signals in the membrane fraction (Figure 3, middle). Following removal of solvent from a chloroform/methanol extraction of ghosts (Figure 3, upper) and resuspension of the residue in buffer by sonication, an EPR line shape corresponding to the partially immobilized species in the original composite spectrum was observed (Figure 3, lower). These results suggest that the immobilized signal is from BSSDP bound to band 3 and/or one of the PAS-staining membrane proteins, and that the partially immobilized labels are bound to phospholipids with primary amino groups in the membrane. This latter signal is well separated from that of membrane protein, however, and can readily be removed from the composite by spectral subtraction as shown in Figure 2.

The labeled proteins giving rise to the immobilized EPR spectrum have been further defined by gel permeation HPLC and by SDS–PAGE. Ghost membranes prepared from BSSDP-labeled erythrocytes were solubilized in 1% SDS, and membrane proteins were separated on a TSK-3000-SW column as shown in Figure 4. Fractions collected from the column were examined by recording EPR spectra of each and computing the integrated signal intensity from the BSSDP present. Spectra were deconvoluted into the slow protein-bound signal and partially immobilized lipid-bound signal, and each was integrated separately. The elution profiles for these signal components are shown by the bar graphs superimposed on the total protein elution profile in Figure 4. Column fractions were also analyzed for membrane proteins on SDS–PAGE as shown in Figure 5. Comparison of the data in Figures 4 and 5 indicates that there was a 1:1 correspondence between integrated signal intensity from the slow component and band 3 protein in the fractions. Moreover, the slow signal was also separated from the major PAS-staining protein band

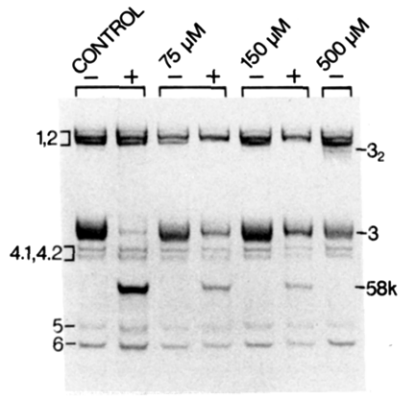

FIGURE 6: Correlation of BSSDP labeling with chymotrypsin cleavage of band 3. Intact erythrocytes were labeled with BSSDP at concentrations ranging from 0 to 500 μM as indicated above, followed by cleavage of band 3 with chymotrypsin [(+) lanes], preparation of ghost membranes, and separation of proteins by SDS–PAGE. Cells in the (−) lanes were carried through parallel incubations but in the absence of chymotrypsin. The two control lanes (left) from cells that were incubated without BSSDP show that chymotrypsin treatment cleaves band 3 such that the Coomassie blue staining intensity essentially disappears from the 95K region of the gel and a new band appears in the 58K region. Labeling with 75 μM BSSDP prior to chymotrypsin treatment results in 70% of the copies of band 3 still migrating in the 95K region of the gel following cleavage [75 μM (−) and (+) lanes] while labeling with 150 μM BSSDP results in 90% of the copies migrating in the 95K region [150 μM (−) and (+) lanes]. Labeling with 500 μM BSSDP (right lane) results in intersubunit cross-linking of 20% of the band 3 monomers with a concomitant shift of a fraction of band 3 from its 95K position to the 190K dimer region (3_2). Band designations on the left are modified from those of Fairbanks et al. (1971). Percentages of staining intensity were estimated from densitometric scans of the individual lanes shown above.

(gel not shown), which was localized in column fractions 14 and 15. The sum of integrated signal intensity recovered in the fractions was greater than 90% of that applied to the column in duplicate trials.

It is significant that spectrin, the major component of the erythrocyte membrane skeleton, was not detectably labeled by treatment of intact erythrocytes (Figure 5). This observation, together with observations that the spin-label moiety of BSSDP bound to intact cells was not chemically reduced by intracellular reductants present in erythrocytes but could be reduced by added exogenous extracellular ascorbate, indicates that BSSDP, like other bis(sulfosuccinimidyl) esters (Staros, 1982), does not penetrate the membrane bilayer.

Domains of Band 3 Labeled by BSSDP. Treatment of intact erythrocytes with bis(sulfosuccinimidyl) suberate, (BS^3), a non-spin-labeled cross-linker with the same reactive groups as BSSDP, results in intrasubunit and intersubunit cross-links in the extracytoplasmic domain of band 3 (Staros & Kakkad, 1983). The intrasubunit cross-link spans the chymotryptic cleavage site in the extracytoplasmic domain (Jennings & Nicknish, 1985). We have now shown that BSSDP also forms an intramolecularly cross-linked product with band 3, which likewise spans the extracellular chymotryptic cleavage site (Figure 6), and that formation of this product precedes intersubunit cross-linking. Lanes 1 [(−) and (+)] are from control erythrocytes with no added BSSDP. Cells prepared for electrophoresis in the (+) lanes were treated with chymotrypsin (Steck et al., 1976) to cleave band 3 prior to preparation of ghost membranes. Cells in the (−) lanes were incubated by the same procedures in the absence of chymotrypsin. Chymotrypsin treatment of intact erythrocytes cleaves band 3 at a single locus into two membrane-associated proteins of M_r 38K and 58K (Steck et al., 1976; Jennings & Passow, 1979; Staros & Kakkad, 1983). The control lanes (left) show that following chymotrypsin cleavage the major protein staining intensity in the 95K region of the gel indicative of band 3 monomers is absent and a new band at 58K appears, indicating that essentially all copies of band 3 are cleaved by this procedure. The 38K peptide is heavily glycosylated and is not readily visualized by Coomassie staining of proteins, though it has been shown to be present in a 1:1 ratio with the 58K fragment (Jennings & Passow, 1979).

Labeling of intact erythrocytes with 75 μM BSSDP prior to chymotrypsin treatment results in 70% of the copies of band 3 migrating in the original 95K region of the gel [Figure 6, 75 μM (+) lane] with the remainder cleaved to produce the 58K fragment. Treatment with 150 μM BSSDP results in 85% of the copies of band 3 migrating in the 95K region of the gel [Figure 6, 150 μM (+) lane]. Increasing the labeling concentration to 500 μM BSSDP results in intersubunit cross-linking of 20% of the copies of band 3 to covalent dimer (Figure 6, 500 μM lane). These estimates are based upon densitometric scans of the gel shown in Figure 6.

It has been shown previously that modification of band 3 with the reactively homologous reagents 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP; Staros & Kakkad, 1983) or BS^3 (Jennings & Nicknish, 1985) does not inhibit band 3 cleavage with chymotrypsin. Thus, the bifunctional active esters must be reacting with amino acid residues on opposite sides of the cleavage site such that the peptides are still covalently attached via the cross-linking reagent. This would explain the shift in band 3 from the 58K to the 95K region of the gels resulting from cross-linking with BSSDP. The residues involved in reaction with the carboxyl termini of the reagent have not as yet been rigorously defined; however, two extracellular lysyl residues have been hypothesized as probable candidates (Jennings & Nicknish, 1985).

The first observed product of BSSDP modification of band 3 is a cross-link that spans the extracellular chymotryptic cleavage site. This is the major product formed at concentrations of reagent below 100 μM with the indicated labeling protocol. Intersubunit cross-linking is observed, but in significant amounts, only at higher concentrations of reagent.

EPR Measurements on Intact Erythrocytes and Ghosts. Two basic conclusions concerning band 3 motion and intersubunit structure can be drawn from the EPR data presented in Figures 2 and 3. First, BSSDP is completely immobilized on the linear EPR time scale when bound to band 3 in intact erythrocytes (Figure 2, lower right), in ghosts (Figure 3, upper), and in stripped membranes (Figure 3, center). The separation between hyperfine extrema (outer peaks) is 67 G in each case. This is the same separation observed in the no-motion limit from bovine serum albumin labeled stoichiometrically with BSSDP (data not shown) and is consistent with the magnitude of the major element of the hyperfine tensor for other doxyl nitroxide probes (Gaffney & McConnell, 1974). It is significant that the rotational motion of the probe remains frozen on the linear EPR time scale ($\tau > 0.1$ μs) in progressing from intact cells to ghosts to stripped ghosts. The rotational motions in all cases must be in the microsecond or longer correlation time range.

Next, the distance between probes bound to intrasubunit binding sites on adjacent band 3 monomers is sufficiently large that they do not interact magnetically. The residual, slow-motion EPR spectrum (Figure 7) from 75 μM labeling of intact erythrocytes does not indicate any measurable dipolar interactions between bound probes even when the majority of copies of band 3 monomer have been labeled with the intramolecular cross-link. Labeling with 75 μM BSSDP results

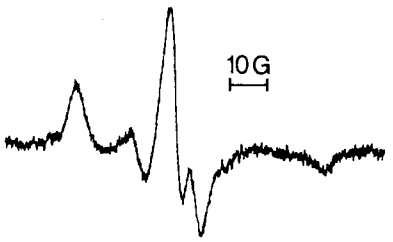

FIGURE 7: Spatial separation of probe bound at the intrasubunit site. Intact erythrocytes were labeled with 75 μM BSSDP as described under Experimental Procedures. This labeling stoichiometry results in covalent modification of band 3 with a ratio of BSSDP/band 3 monomer of 0.7/1.0. The residual EPR spectrum shown was obtained by digital subtraction of the unbound component (Figure 2, upper) and partially immobilized component (Figure 2, middle). This line shape is indistinguishable from those obtained at lower labeling stoichiometries, where only a fraction of the copies of band 3 have been modified, and is characteristic of a spatially isolated and immobilized doxyl nitroxide probe.

in intrasubunit cross-linking of 70% of the band 3 monomers present with no observable intersubunit dimer formation (Figure 6). It should be noted that this represents a lower limit for stoichiometry of labeling since only probes bound across the chymotryptic site would prevent band 3 from migrating as proteolytic fragments in the gels. It is possible that some labels may cross-link residues within the 58K or 38K peptides and thus not prevent cleaved band 3 from migrating as lower molecular weight fragments. Evidence for minor labeling of these fragments with [^{14}C]BS^3 has recently been observed (unpublished results).

A high labeling stoichiometry is likewise indicated by comparing the molar concentration of spin-label in the immobilized environment with the molar concentration of band 3 monomers in the sample. The concentration of label bound to protein in Figure 7 was calculated by comparing integrated signal intensity with similar measurements made on standard samples of bovine serum albumin labeled with a variety of concentrations of 16-doxylstearate in the same buffer (not shown). Spin concentration determinations indicated that 1.0 ± 0.1 BSSDP labels were bound per band 3 monomer in three separate trials. Calculation of stoichiometry was based upon 1.2×10^6 copies of band 3 per erythrocyte (Fairbanks et al., 1971; Fröhlich, 1982) and the number of cells per milliliter determined in a Coulter counter. This number represents an upper limit for labeling since all of the slow-motion signal has been assigned to band 3 labeling. The true ratio of BSSDP/band 3 must be between 0.7 (from chymotryptic cleavage data, Figure 6) and 1.0 based upon these observations.

Band 3 Rotational Mobility in Intact Erythrocytes and Ghost Membranes. Employing the labeling protocols outlined above, we have examined the rotational motion of BSSDP-labeled band 3 in intact erythrocytes and in ghost membrane preparations. Figures 2 and 3 demonstrate that band 3 mobility in intact cells and ghost membranes is frozen on the EPR time scale, indicating a motional frequency in the microsecond or longer correlation time range. Thus, we have employed ST-EPR (Hyde & Dalton, 1972; Hyde & Thomas, 1973), a technique sensitive to nitroxide probe dynamics in the 0.1-μs to 1-ms correlation time range (Thomas et al., 1976), to measure and compare membrane protein mobility in these systems.

The ST-EPR spectra in Figure 8 demonstrate a significant increase in the rotational dynamics of band 3 upon hypotonic lysis of intact erythrocytes. The upper spectrum was obtained from intact erythrocytes at 50% hematocrit in isotonic phosphate-buffered saline. First-order analysis of this spectrum

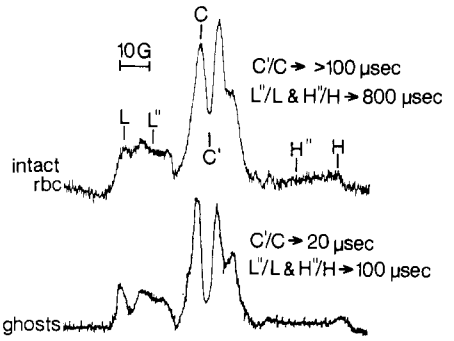

FIGURE 8: Rotational dynamics of BSSDP-labeled band 3 in intact erythrocytes and ghost membranes. ST-EPR spectra were recorded on intact erythrocytes (upper) and ghost membranes (lower) as described under Experimental Procedures. The positions where motionally sensitive ratio parameters L''/L, C'/C, and H''/H are measured are shown in the upper display. Comparison of these parameters measured from the experimental spectra shown with plots of the same parameters determined from ST-EPR spectra recorded from spin-labeled hemoglobin in various glycerol/buffer solutions (Thomas et al., 1976) allowed determination of effective correlation times for reorientation of band 3. The correlation times determined by each ratio parameter are shown in the panel with the corresponding spectrum. The results demonstrate a significant increase in motion of band 3 in ghost membranes as compared to intact cells. The spectra shown were recorded at 20 °C. The rotational diffusion data from these experiments are tabulated in Table I along with data from intact cells and ghost membranes at 2 and 40 °C.

Table I: Effective Rotational Correlation Times for Band 3 Protein in Intact Erythrocytes and Ghost Membranes vs. Temperature[a-c]

		erythrocytes		ghosts	
T (°C)	ratio parameters	τ	D (s^{-1})	τ	D (s^{-1})
2	L''/L	>1 ms	<150	700 μs	238
	C'/C	>100 μs	<1700	60 μs	2800
	H''/H	>1 ms	<150	700 μs	238
20	L''/L	800 μs	208	100 μs	1700
	C'/C	>100 μs	<1700	20 μs	8300
	H''/H	800 μs	208	100 μs	1700
40	L''/L	300 μs	555	80 μs	2100
	C'/C	20 μs	8300	8 μs	20000
	H''/H	300 μs	555	80 μs	2100

[a] The ratio parameters were measured from experimental V_2' spectra as indicated in Figure 8. Effective rotational correlation times were estimated by comparison of these parameters with standard curves of the same parameters from a model system study of hemoglobin in glycerol/buffer solutions (Thomas et al., 1976). [b] Diffusion coefficients were calculated from correlation times by $D = 1/(6\tau)$. [c] Values listed as ">" are out of the range of sensitivity of that parameter for estimating τ.

by comparison of motionally sensitive ratio parameters, L''/L, C'/C, and H''/H, with plots of these parameters obtained from a model system study of spin-labeled hemoglobin in glycerol/buffer solutions (Thomas et al., 1976) indicated that band 3 was reorienting in the membrane with an effective correlation time of 0.1–0.8 ms at 20 °C, depending on the ratio parameter employed (Table I). The spectral contribution from the lipid component (Figure 2, middle right) has been digitally subtracted from each of the displays in Figure 8 so that band 3 motion is the principal determinant of the observed line shapes.

Hypotonic lysis of these labeled erythrocytes in the presence of ascorbic acid oxidase to prevent chemical reduction of the spin-label, followed by removal of cytosolic components by repeated washing of cells, resulted in recovery of white ghost membranes which retained >95% of the integrated signal intensities from the slow- and intermediate-motion spin-label signals (Figure 3, upper). ST-EPR measurements on these ghost membranes (Figure 8, lower) indicated that the motion of band 3 had increased significantly when compared to intact

cells (Figure 8, upper). Again, first-order analysis by ratio parameter comparisons indicated a motional frequency of 20 μs to 0.1 ms at 20 °C, depending on the ratio parameter analyzed (Table I).

ST-EPR measurements on intact cells and ghost membranes made at temperatures of 2, 20, and 40 °C indicated a monotonic increase in mobility of labeled band 3 with increased temperature (Table I). At all temperatures the mobility of band 3 was significantly increased in ghosts when compared to intact cells. The significance of the motional differences presented is evident by the similarity of results obtained from ghosts at 2 °C with those from intact cells at 20 °C.

Discussion

The spin-labeling reagent BSSDP developed in this work has provided new opportunities for examing the rotational diffusion of the integral membrane protein band 3 in intact human erythrhocytes. BSSDP is also potentially useful as a general spin-labeling reagent for a variety of proteins including integral proteins in the plasma membrane of other cell types as well as purified soluble proteins. The bifunctional character provides a tight coupling of motion of the probe to that of the protein being labeled, a critical objective when interactions of proteins to form macromolecular assemblies are being investigated. In this regard, BSSDP is similar to the bifunctional protein modification reagents developed by Gaffney et al. (1983), which also provided a strong coupling between motions of the probe and protein for both soluble and membrane-bound proteins (Willingham & Gaffney, 1983).

In addition to imparting high reactivity with nucleophiles (Anjaneyulu & Staros, 1986), the two *N*-hydroxysulfosuccinimide moieties of BSSDP serve a number of other important functions. First, they provide affinity groups for directing the probe to positively charged regions of a protein, such as groups of lysyl residues. Indeed, the non-spin-labeled homologue, BS^3, has been shown to compete for the stilbenedisulfonate site in band 3 (Jennings & Nicknish, 1985; Jennings et al., 1985). Next, the strong anionic character of the two sulfonate groups makes the reagent impermeant to lipid bilayers, allowing labeling exclusively at the exofacial surface of cells (Staros, 1982) and thus minimizing complications arising from labeling of cytosolic proteins in addition to integral membrane proteins. They also impart high water solubility (Staros, 1982), thus overcoming the limited solubility problems frequently encountered with *N*-hydroxysuccinimide reagents for biological studies. Finally, since the anion moieties are the leaving groups resulting from addition of nucleophiles (Staros, 1982), potential for maintenance of biological function of proteins that must bind anions is provided. Functional characterization of the labeled erythrocytes in this work indicated 70% of the control level of uptake of $^{35}SO_4^{2-}$ was maintained following covalent modification with 50 μM BSSDP (data not shown). This is in agreement with the work of Jennings et al. (1985), who observed maintenance of anion transport with only a shift in pH optimum following modification of band 3 in intact erythrocytes with the structurally homologous probe, BS^3.

The symmetry of BSSDP optimizes chances for a unique magnetic orientation of the spin-label relative to the protein being labeled, an important criterion for preserving sensitivity to rotational anisotropy of a spin-labeled membrane protein (Beth et al., 1983; Robinson & Dalton, 1980). The rate of addition of nucleophilic residues such as the ϵ-amino group of lysine greatly exceeds the rate of hydrolysis for a variety of sulfosuccinimidyl esters (Anjaneyulu & Staros, 1986), thus minimizing chances for only one end of the reagent being covalently attached to the protein being labeled.

The bifunctional character of BSSDP provides information on distances between residues that it bridges, as do other cross-linking reagents. Nucleophiles capable of approaching each other within 10 Å can be spanned by cross-link formation with the carboxyl termini of BSSDP. The presence of the spin-label moiety also provides a convenient marker for examining longer range structural arrangements through analysis of interactions between probes bound to neighboring protein monomers. The EPR data presented on spin-labeled band 3 (Figures 2, 3, and 7) have provided information on the spatial arrangement of the BSSDP binding domains on adjacent band 3 monomers and thus on oligomeric structure.

There is general agreement that band 3 is dimeric in the erythrocyte membrane (Yu & Steck, 1975; Staros & Kakkad, 1983; Macara & Cantley, 1983; Jennings & Nicknish, 1985) although association of dimers to form tetramers or higher order structures has been observed in ghosts (Weinstein et al., 1980). Two different segments of the protein have been shown to be areas of contact between monomers. One area of contact is in the cytoplasmic, *N*-terminal domain, where a disulfide bond can be formed between two subunits by oxidation of endogenous thiols with Cu^{2+} *o*-phenanthroline (Steck, 1972; Steck et al., 1976). A second area of contact is in the extracytoplasmic domain, where a cross-link can be formed between two subunits by using membrane-impermeant cross-linking reagents (Staros et al., 1981; Staros, 1982; Staros & Kakkad, 1983). Jennings and Nicknish (1985) combined these procedures to show that the same two monomers were cross-linked at the cytoplasmic and exofacial domains. Since we do not observe any dipolar interaction between BSSDP probes bound to adjacent monomers in the membrane dimer, these domains (or, technically, the nitroxide moieties bound in these domains) must be at least 14 Å from each other.

The magnitude of dipolar interactions between immobilized nitroxide spin-labels is critically dependent on the angles and distance relating the interacting probes (Carrington & McLachlan, 1967). Analysis of the EPR line shape observed from interacting probes bound to proteins can yield detailed intersubunit structural information (Beth et al., 1984). If no interactions are observed, then a minimum distance between bound probes can be established. Minimum sensitivity to radial separation would occur if the *Z* axes of two interacting probes were each oriented at the magic angle (54.7°) with respect to the interelectron axis (Carrington & McLachlan, 1967). Under these circumstances, the outer extrema of the EPR spectrum are not split by dipolar interaction with the neighboring probe. Even in this case, discernible extra structurings in EPR line shapes appear in the central region of the spectrum due to dipolar interactions as the probes approach each other within 14 Å (unpublished calculations). We can thus define a minimum distance between the nitroxide moieties of BSSDP labels bound to adjacent band 3 monomers as 14 Å. Additional proteolytic cleavages of band 3 and planned sequence determinations on the resulting peptides will allow the residues bridged by BSSDP to be defined within the primary structure and, hence, constraints on the spatial arrangement of these specific domains to be established.

The domains of band 3 labeled by BSSDP are of particular interest in light of recent work which demonstrated that BS^3 and 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) compete for a common or overlapping binding site on band 3 (Jennings & Nicknish, 1985; Jennings et al., 1985). The similarities in band 3 labeling profiles with BS^3 (Staros, 1982; Staros & Kakkad, 1983) and the structurally homologous spin-labeled cross-linker

BSSDP (Figure 6) suggest that these reagents may be bridging the same residues and, thus, that BSSDP may also be bound in or near the DNDS site. There are a number of open questions concerning the nature and disposition of the stilbenedisulfonate binding sites on adjacent band 3 monomers in the erythrocyte. Cooperativity in binding has been observed under some conditions (Dix et al., 1979) but absent under others (Rao et al., 1979). Boodhoo and Reithmeier (1984) showed that monomeric band 3 purified from the erythrocyte and conjugated to a solid support could not bind stilbenedisulfonates while dimeric species in dilute detergent bound them with high affinity. This latter study suggests the possibility of inhibitor binding sites at monomer–monomer interfaces. If our spin-label reagent is binding to the same site as the stilbenesulfonate site, then the observation of a substantial distance (>14 Å) between these sites is of considerable interest.

The results obtained from ST-EPR studies on the rotational diffusion of band 3 in intact erythrocytes and ghost membranes (Figure 8) have provided evidence that significant alterations in interactions between membrane proteins occur as a result of cell lysis. Changes in rotational diffusion of band 3 cannot be attributed to alterations in the bulk bilayer since the ordering of membrane lipids, as monitored by spin-labeled stearic acid probes, is very nearly the same in intact erythrocytes and ghost membranes (unpublished observations). Before embarking on further discussions of possible factors that determine band 3 rotational diffusion, we should first comment on the significance of the motional differences presented in Figure 8 and Table I.

We observed a substantial and reproducible change in ST-EPR line shape, which was manifested throughout the spectral display. All three motionally sensitive ratio parameters (Table I) were simultaneously altered, indicating as much as a factor of 8 (from L''/L and H''/H, Figure 8) decrease in "effective" correlation time resulting from cell lysis and preparation of ghost membranes. Since these three parameters are differentially sensitive to rotational motions about the three unique magnetic axes (principal axis system of the nitroxide) of the probe (Thomas et al., 1976; Robinson & Dalton, 1980; Beth et al., 1983), a simple rearrangement of the orientation of the label relative to the symmetry axis (membrane normal axis) of the protein would not account for the data. Such a rearrangement would have opposite effects on the apparent motional rates determined from the extrema regions (L''/L and H''/H), which are sensitive only to motional averaging of the nitroxide Z axis with X and Y axes, and the central region (C'/C), which is maximally sensitive to motional averaging of the nitroxide X and Y axes (Thomas et al. 1976; Robinson & Dalton, 1980). Therefore, the rotational frequency averaged over all copies of band 3 present is significantly increased in the ghost membrane preparations relative to intact erythrocytes.

The correlation times presented in Table I were determined by ratio parameter comparisons with the same parameters measured from spin-labeled hemoglobin in glycerol/buffer solutions (Thomas et al., 1976). Significantly different correlation times are consistently estimated from the C'/C parameter than from L''/L and H''/H. This may be a result of the anisotropic diffusion of band 3 in the membrane. The rotational diffusion of hemoglobin in glycerol/buffer would be expected to approximate isotropic reorientation based upon hydrodynamic shape. The correlation times in Table I, therefore, should be interpreted as "effective correlation times" for the true reorientation times of band 3. The data presented provide a qualitative observation of a significant difference in rotational dynamics between this integral membrane protein in intact cells and ghost membranes but do not define true correlation times for rotation of band 3.

We have recorded ST-EPR spectra on intact erythrocytes labeled with BSSDP over the range of temperatures from 2 to 40 °C (Table I, Figure 8). The temperature dependence observed supports a tight coupling of the motion of BSSDP with that of a large segment of band 3 protein. Data recorded at 2 °C (Table I) indicate that the probe is frozen on the ST-EPR time scale ($\tau > 1$ ms). This agrees with the optical data of Nigg and Cherry (1979b) from eosin–maleimide labeled band 3 in ghost membranes. In this study the authors observed a strong temperature dependence of band 3 rotation that approached the no-motion limit on the time scale of their experiments at temperatures near 0 °C. We observe a steep monotonic increase in rotational motion through the temperature range from 2 to 40 °C. This result is consistent with monitoring mobility of a large segment of protein rather than a local mode independent motion of the probe relative to band 3.

A number of variables must be examined before a rigorous explanation of the observed differences in mobility of band 3 between intact cells and ghost membranes can be established. These include the effects of ionic strength, cytoskeletal integrity, and noncytoskeletal proteins that bind to band 3. Studies directed at this objective are now feasible given the development of a probe that can monitor the motion of band 3 under the full range of conditions from intact cells to membrane preparations with selected protein–protein interactions disrupted.

Nigg and Cherry (1979b) showed that their optical data could be fit by assuming two motional populations of band 3 in ghost membranes. In a subsequent experiment (Nigg & Cherry, 1980), they interpreted these populations as arising from band 3 oligomers bound to membrane skeletal proteins exhibiting slow (millisecond) rotational motion and a second population not directly bound to this network exhibiting faster rotational motion. This model is supported by estimates of the number of copies of ankyrin (Bennett & Stenbuck, 1979b), a protein that connects band 3 directly to the membrane skeleton, and the number of copies of band 3 oligomers detected by freeze–fracture electron microscopy (Weinstein et al., 1980). However, a model based on the stoichiometry of ankyrin to band 3 may significantly underestimate the proportion of anion channels that are motionally restricted by interaction with the cytoskeleton. It does not, for example, take into account band 3 dimers associated with glycophorin (Pinto da Silva & Nicholson, 1974; Nigg et al., 1980), which, in turn, is reported to be linked to the spectrin–actin network via band 4.1 (Anderson & Lovrien, 1984).

Interpretation of our data in terms of a simple two-site model leads to the hypothesis that disruption of cells results in a decrease in the number of band 3 oligomers that are interacting at any instant with the membrane skeleton and thus exhibiting restricted rotational mobility. This is undoubtedly an oversimplified model in that many interactions of band 3 with membrane proteins, including self-association, will determine the rotational diffusion rate. We emphasize that our results are based on measurements that reflect a motional average over all spin-labeled copies of band 3. Motional differences detected between intact cells and ghosts must be interpreted as a shift in the motional average.

In summary, we have developed a spin-labeling reagent for specific covalent modification of band 3 protein in intact erythrocytes. Results from EPR and ST-EPR investigations

carried out with this reagent have indicated that significant alterations in protein–protein interactions between intact cells and ghost membranes may exist. These differences must be addressed before protein dynamic measurements (either rotational or translational) made on ghost membranes can be reliably extrapolated to expected behavior in the intact cell. Future studies with this probe and related homologues, which are being synthesized, promise to supplement our current understanding of protein–protein interactions in cell membranes.

ACKNOWLEDGMENTS

Mass spectral analyses of compounds were kindly provided by Dr. B. Sweetman and were supported by RR 01688 and RR 05424. Technical assistance by D. Swingle and S. Juliao is gratefully acknowledged. We thank Drs. R. Whitesell and N. Abumrad for assistance with sulfate-transport assays.

Registry No. BSSDP, 102260-45-7; BSSDP (hydroxylamine), 102260-49-1; 4-doxylpimelate, 102260-46-8; diethyl 4-oxopimelate, 6317-49-3; diethyl 4-doxylpimelate, 102260-47-9; 4-doxylpimelic acid (hydroxylamine), 102260-48-0; *N*-hydroxysulfosuccinimide, 82436-78-0.

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