Identification of the Eosinyl-5-maleimide Reaction Site on the Human Erythrocyte Anion-Exchange Protein: Overlap with the Reaction Sites of Other Chemical Probes†

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Received March 19, 1990; Revised Manuscript Received May 16, 1990

ABSTRACT: The anion-exchange protein (band 3) reaction site in human erythrocytes for the fluorescent/phosphorescent probe eosinyl-5-maleimide (EMA) has been identified. Proteolytic dissection of band 3 in situ indicated that EMA reacts with the membrane-spanning M_r 17K peptide produced by chymotrypsin cleavage of band 3 in *intact* erythrocytes followed by removal of the cytoplasmic domain by mild trypsin digestion of ghost membranes. Sequencing of the major eosin-labeled peptide obtained from HPLC purification of an extensive chymotrypsin digest of purified M_r 17K peptide allowed assignment of the covalent reaction site for EMA to lysine-430 of the human erythrocyte protein [Tanner et al. (1988) *Biochem. J. 256*, 703–712]. Hydropathy plots based upon the primary structure of the protein [Lux et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9089–9093] suggest that this residue is in an extracellularly accessible loop connecting membrane-spanning segments 1 and 2 of native band 3 in the erythrocyte membrane. Inhibition of sequential labeling of *intact* erythrocytes by pairs of chemical probes including EMA, the anion transport inhibitor 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2-DIDS), and the reactively bifunctional spin-label bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate (BSSDA) has also been investigated. Each of these reagents affinity labels band 3 when added separately to a suspension of *intact* human erythrocytes by formation of one or more stable covalent bonds. Prelabeling of *intact* erythrocytes with EMA reduced subsequent labeling of band 3 by H_2-DIDS by approximately 95% and by BSSDA by 90%. Similarly, prelabeling with H_2-DIDS reduced subsequent labeling of band 3 by EMA by over 90%, and BSSDA prelabeling reduced EMA labeling by approximately 95%. Therefore, though having widely divergent chemical structures and protein modification reactivities, each of these negatively charged reagents may be competing for reaction with spatially overlapping sites on band 3 which are accessible from the extracellular space.

The anion-exchange protein (band 3) is an abundant intrinsic membrane protein of the human erythrocyte which catalyzes the one-for-one exchange of chloride for bicarbonate across the lipid bilayer [for reviews, see Knauf (1979), Parrow (1896), Jay and Cantley (1986), and Jennings (1989)]. Band 3 is composed to two functionally distinct domains: a M_r 52K transmembrane domain (Steck et al., 1976) which traverses the bilayer multiple times (Kopito & Lodish, 1985; Tanner et al., 1988; Lux et al., 1989), thereby forming the anion-exchange pathway (Lepke & Passow, 1976), and a M_r 43K cytoplasmic domain extending into the interior of the cell (Steck et al., 1976), which forms a physical link with the underlying membrane skeleton via the bridging protein ankryin (Bennett, 1978; Bennett & Stenbuck, 1980) as well as providing a membrane binding site for cytosolic proteins including hemoglobin (Shaklai et al., 1977), glyceraldehyde-3-phosphate dehydrogenase (Kant & Steck, 1973), and aldolase (Karadsheh & Uyeda, 1977).

Elucidation of details of band 3 structure, function, and motional dynamics has been facilitated by data obtained from a variety of exogenous chemical probes. Early studies which led to identification of band 3 as the anion-exchange protein in human erythrocytes (Cabantchik & Rothstein, 1974) as well as numerous subsequent studies on kinetic and mechanistic aspects of anion-exchange function have relied heavily upon the use of stilbenedisulfonate inhibitors of anion exchange. Two members of this family of inhibitors, 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS)[1] and its dihydro derivative 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2-DIDS), have proven to be extremely useful markers because of their high specificity for covalent modification of band 3 in *intact* erythrocytes (Cabantchik & Rothstein, 1974; Jennings & Passow, 1979).

More recently, spectroscopic probes have been employed to examine dynamic and structural properties of band 3. Cherry and co-workers have shown that eosinyl-5-maleimide (EMA) affinity labels band 3 in *intact* erythrocytes (Nigg & Cherry, 1979). Following preparation of ghost membranes from EMA-labeled cells, band 3 rotational diffusion has been observed in intact ghosts and in ghost membranes having specific protein–protein interactions disrupted (Nigg & Cherry, 1979, 1980) by measuring flash-induced transient dichroism of the eosin probe (Cherry et al., 1976). These studies, and those of others (Tsuji et al., 1988), have suggested that a subpopulation of band 3 is restricted in rotational motion via interactions involving its cytoplasmic domain and membrane

† This work was supported in part by grants from the National Institutes of Health (HL34737 and RR04075).

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[1] Abbreviations: EMA, eosinyl-5-maleimide, EMA/17K, EMA-labeled M_r 17K transmembrane segment of band 3; BSSDP, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate; BSSDA, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer EPR; DATD, *N*,*N*′-diallyltartardiamide; H_2-DIDS, 4,4′-diisothiocyanodihydrostilene-2,2′-disulfonic acid; HPLC, high-performance liquid chromatography; RP-HPLC, reverse-phase HPLC; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; TFA, trifluoroacetic acid; PTH-amino acids, phenylthiohydantoin-derivatized amino acids.

0006-2960/90/0429-8283$02.50/0

skeletal proteins. Clague et al. (1989) have recently suggested that this slow rotational subpopulation arises from small aggregates of band 3. The great flexibility of the membrane skeletal components (especially spectrin) has been proposed to account for the lack of a major effect of the membrane skeleton per se on band 3 rotational motion (Clague et al., 1989). Macara et al. (1983) showed that EMA covalently bound to band 3 was more accessible to Cs^+ quenching of its fluorescence from the cytoplasmic side of the membrane than from the extracellular side, suggesting that the eosin probe, though added to *intact* cells extracellularly, became exposed to the cytoplasmic surface of the membrane.

Other probes which have proven useful for studies of band 3 structure and dynamics are the reactively bifunctional active ester spin-labeling reagents bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate (BSSDP; Beth et al., 1986) and bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate (BSSDA; Anjaneyulu et al., 1989). These reagents, which were designed based upon the favorable reactivity properties and membrane impermeance of non-spin-labeled bis(sulfo-*N*-succinimidyl) cross-linking reagents (Staros, 1983, 1988), affinity label band 3 in *intact* erythrocytes by formation of a stable cross-link which spans the extracellular chymotrypsin cleavage site (Beth et al., 1986; Anjaneyulu et al., 1989). Electron paramagnetic resonance (EPR) and saturation-transfer EPR (ST-EPR) studies which have been carried out using BSSDP- or BSSDA-labeled band 3 have provided new insights into the rotational dynamics of band 3 in *intact* erythrocytes and ghost membranes (Beth et al., 1986; Anjaneyulu et al., 1989) as well as on the intermolecular distance between BSSDP binding sites on adjacent monomers of band 3 dimers and the stability of the band 3 dimer in *intact* erythrocytes (Anjaneyulu et al., 1988).

The spectroscopic studies outlined above require that one infers structural and/or dynamic properties of band 3 from spectral information provided by the probe molecule being employed. Therefore, it is of great importance to know (a) if the spectroscopic probe reacts in a 1:1 stoichiometry with band 3 and, if so, that all the probe molecules are labeling a single locus on band 3 and (b) the location of the reaction site(s) with regard to the overall structure of band 3 in the membrane. Moreover, if experimental data from complementary spectroscopic studies such as fluorescence/phosphorescence and ST-EPR are to be meaningfully compared, it is necessary to know which domains of band 3 are being monitored by the probes utilized for each technique. In the present work, we have investigated the labeling of band 3 in *intact* erythrocytes by EMA and have shown that lysine-430 of the human protein (Tanner et al., 1988; Lux et al., 1989) is the major site for covalent modification. In separate experiments, we have investigated the effect of EMA labeling of *intact* erythrocytes on subsequent labeling by BSSDA or by $[^3H_2]$DIDS as well as the effects of labeling with BSSDA or $[^3H_2]$DIDS on subsequent labeling by EMA. These sequential labeling studies have indicated that though the amino acid residues involved in reaction of these probes may be different, each one effectively inhibits labeling of band 3 by any one of the others added subsequently, suggesting that they compete for one or more spatially overlapping reaction site(s). Portions of this work have been published in the form of an abstract (Cobb et al., 1990).

Experimental Procedures

Protein concentrations of SDS-solubilized ghosts and purified peptides were determined by using the Pierce Chemical Co. BCA assay (BSA standard). BSSDA was synthesized as described by Anjaneyulu et al. (1989) and $[^3H_2]$DIDS was prepared from $[^3H_2]$DADS as described by Cabantchik and Rothstein (1974). EMA was purchased from Molecular Probes, Inc. Ascorbate oxidase, TLCK-treated α-chymotrypsin, TPCK-treated trypsin, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. Reagents used for peptide sequencing were purchased from and prepared according to Applied Biosystems. SDS–PAGE chemicals were electrophoresis grade and were purchased from Bio-Rad. All other reagents and chemicals were purchased from Fisher Scientific or Aldrich Chemical Co. and were of reagent grade or better quality.

Preparation of EMA-Labeled Band 3 Peptides. Fresh whole blood from normal volunteers was drawn into heparinized vacutainer tubes and cooled on ice (all subsequent steps were performed at 0–4 °C unless otherwise indicated). Erythrocytes were collected by centrifugation and washed 4 times with 4–5 volumes of phosphate-buffered saline (5PBS7.4; 5 mM sodium phosphate/150 mM sodium chloride at pH 7.4). The *intact* erythrocytes were labeled at a ratio of 1 volume of 0.5 mg/mL EMA (674 μM; freshly prepared in 5PBS7.4) to 5 volumes of cells at 50% hematocrit by incubation in the dark at room temperature for 45 min. The excess EMA was removed by washing the cells with 4 volumes of 5PBS7.4 containing 0.2 % (w/v) BSA followed by three washes with 5PBS7.4 alone.

The labeled, *intact* erythrocytes were treated with chymotrypsin to cleave the band 3 protein into M_r 38K and 58K polypeptides. Packed EMA-labeled erythrocytes were diluted with an equal volume of 5PBS7.4 and treated with 75 μg/mL (final concentration) chymotrypsin at 37 °C for 30 min. The reaction was stopped by addition of 2 volumes of ice-cold 5PBS7.4 containing 30 μg/mL PMSF (added from a stock solution in ethanol). The erythrocytes were collected by centrifugation and washed twice with 5PBS7.4, followed by hemolysis in 20 volumes of 5 mM sodium phosphate, pH 8.0 (5P8.0). The erythrocyte membranes (ghosts) were pelleted by centrifugation and washed 3 times with 5P8.0 to complete the removal of cytosolic proteins. After hemolysis, all subsequent operations were conducted under reduced light conditions (fluorescent room lights off and windows covered) in order to minimize photooxidative cross-linking of membrane proteins (Nigg et al., 1979). The ghosts were treated with 100 μg/mL trypsin in 5P8.0 buffer at room temperature for 30 min to cleave the M_r 58K band 3 polypeptide into a M_r 17K integral membrane segment and a M_r 43K soluble segment. The reaction was stopped, and peripheral membrane proteins were stripped from the ghosts by addition of 20 volumes of 0.1 N NaOH and centrifugation to pellet the ghosts. The ghosts were then washed once with 20 volumes of 5 mM sodium phosphate (pH 5.0) and once with 20 volumes of 5P8.0, dissolved in electrophoresis sample buffer [final concentrations: 125 mM Tris, 1% (w/v) SDS, 10 mM DTT, and 15% (w/v) sucrose, pH 8.8], and purged with argon for 2–3 min before freezing at −70 °C.

The EMA-labeled 17K peptide (EMA/17K) was purified by SDS–PAGE on a 10–20% gradient slab gel with a 4% stacking gel using the Tris/glycine/SDS buffer system of Laemmli (1970). The pink EMA/17K band was excised from the gel and electroeluted with an Isco Model 1750 electrophoretic concentrator (50 mM ammonium bicarbonate electrode buffer, 5 mM ammonium bicarbonate/0.05% SDS sample chamber buffer). The electroeluted EMA/17K peptide solution was purged with argon and stored at −70 °C until further cleavage with CNBr or chymotrypsin. The EMA/17K

peptide and its cleavage products were quantitated by absorption spectrophotometry based on their eosin content (OD_{530}, ϵ = 96 $mM^{-1} \cdot cm^{-1}$; Tsuji et al., 1988).

The CNBr cleavage was done in a 1.5-mL microfuge tube containing 500 μL of purified EMA/17K peptide (in 0.1% SDS, approximately 3 nmol), 200 μL of TFA, 50 μL of water, and 2.5 mg of CNBr. The reaction mixture was purged with argon for 2 min, sealed, and then incubated at room temperature in the dark for 24 h. The reaction was terminated by diluting the mixture with 10 mL of water and lyophilizing twice. The dried peptide mixture was dissolved in electrophoresis sample buffer and separated on a 15–20% gradient denaturing gel, and the eosin-containing band was excised from the gel and electroeluted as described above. The excess SDS and buffer ions were eliminated from the purified EMA-labeled peptide solution by buffer exchange with a total of 20 volumes of 0.01% SDS via a 10-mL Amicon ultrafiltration cell fitted with a PM-10 membrane, followed by lyophilization. In all ultrafiltration procedures, the SDS concentration was kept 5–10-fold lower than its critical micellar concentration to prevent concentration of SDS in the resulting peptide solution. This peptide was suitable for partial sequencing as described below.

In order to obtain a smaller EMA-labeled peptide suitable for direct sequencing of the EMA reaction site, the purified EMA/17K was concentrated and equilibrated with a chymotryptic digestion buffer consisting of 5P8.0, 0.1% (w/v) SDS, 0.1% (w/v) $C_{12}E_8$, and 0.1% (v/v) β-mercaptoethanol (Casey & Reithmeier, 1989; personal communication) by buffer exchange using a 10-mL Amicon ultrafiltration cell fitted with a PM-10 membrane. The EMA/17K peptide solution (approximately 7.5 nmol of EMA/17K in 1 mL) was heated to 60 °C for 10 min and then cooled to 37 °C. Chymotrypsin was added in three aliquots of 75 μg/mL each (final concentration) at t = 0, 2, and 18 h, with a total digestion time of 24 h at 37 °C. The reaction was stopped by addition of SDS to a final concentration of 1% and incubating at 100 °C for 5 min. The peptides were separated by reverse-phase HPLC on an Alltech C18RP 4.6 × 250 mm column using a linear gradient from 95% water/0.1% TFA (solvent A) and 5% acetonitrile/0.1% TFA (solvent B) to 10% solvent A/90% solvent B and detected by absorbance monitoring at 215 nm. Peptide peaks were collected, lyophilized, and redissolved in 10 mM ammonium hydroxide, and eosin-containing peptides were identified spectrophotometrically. The main EMA-labeled peptide (approximately 85–90% of the total eosin eluted from the column) was lyophilized, redissolved in 250 μL of water, and relyophilized.

Sequencing of all EMA-labeled peptides was performed on an Applied Biosystems Model 475 liquid-phase sequenator. Each purified peptide (between 300 and 1000 pmol in 30 μL of water) was applied to a precycled polybrene-coated filter and allowed to dry before assembly of the reaction chamber. Standard sequencing protocols from Applied Biosystems were employed. PTH-amino acids were chromatographed and analyzed via an on-line Applied Biosystems 120A amino acid analyzer.

Labeling of Intact Erythrocytes with EMA and BSSDA. Erythrocytes were labeled with EMA as described above in all labeling experiments. In some experiments, erythrocytes were also labeled with the bifunctional nitroxide spin-probe BSSDA either before or after labeling with EMA. The BSSDA labeling procedure was essentially the same as that of Anjaneyulu et al. (1989). Erythrocytes (nonlabeled or prelabeled with EMA) equilibrated with 106 mM sodium phosphate, pH 7.4, were diluted to 50% hematocrit with the same buffer. An aliquot of freshly prepared BSSDA stock solution was added to a final concentration of 50 μM, and the cells were then incubated at 20 °C for 15 min. The reaction was quenched by addition of 3 volumes of sodium phosphate buffer containing 0.5% (w/v) BSA followed by incubation for 30 min at 20 °C. The erythrocytes were then washed 3 times with 6 volumes of ice-cold 5PBS7.4, followed by EMA labeling or by hemolysis in 5P8.0 containing 1 μg/mL ascorbate oxidase to minimize chemical reduction of the spin-label moiety. EPR spectra were recorded on BSSDA-, BSSDA/EMA,[2] and EMA/BSSDA-labeled ghosts as described below, followed by dissolution of the ghost samples in SDS (final concentration of 1% w/v). EMA labeling was quantitated by absorption spectrophotometry on the dissolved ghost samples, and 1.0-mL aliquots (approximately 3.5 mg of membrane protein) of the labeled ghosts were separated by HPLC gel filtration on a 7.5 × 600 mm TSK-4000 column as previously described (Anjaneyulu et al., 1989) except the flow rate was lowered to 0.8 mL/min. The column eluent was monitored at 280 and 528 nm, and 1.0-min fractions were collected. EPR spectra were recorded on the fractions, the fast- and slow-motion components were separated by digital subtraction, and the relative signal intensity was quantitated by comparison of the center EPR peak heights. Dissolved labeled ghost and HPLC fraction proteins were also analyzed by SDS–PAGE.

Labeling of Intact Erythrocytes with EMA and H_2-DIDS. Chymotrypsin-treated control or EMA-labeled chymotrypsin-treated erythrocytes were diluted to 20% hematocrit with 5PBS7.4, [$^3H_2$]DIDS was added to a final concentration of 15 μM, and the cell suspension was incubated at 37 °C for 30 min. These reaction conditions facilitate the covalent reaction between one isothiocyano reactive group of [$^3H_2$]DIDS and the M_r 58K chymotryptic band 3 polypeptide. Three volumes of 5PBS7.4 containing 0.5% BSA were then added, and the erythrocytes were collected by centrifugation. The cells were washed 3 times with ice-cold 5PBS7.4 to eliminate unbound [$^3H_2$]DIDS and BSA, and an aliquot of these [$^3H_2$]DIDS-labeled control erythrocytes was labeled with EMA as described above. Another aliquot of the [$^3H_2$]DIDS control cells and the EMA-prelabeled cells was equilibrated with cold 150 mM $NaHCO_3$ (pH 9.5) buffer by repeated centrifugation and resuspension of the erythrocytes in this buffer. The packed erythrocytes were then diluted to 33% hematocrit and incubated at 37 °C for 1 h to facilitate formation of the covalent cross-link between the [$^3H_2$]DIDS-labeled M_r 58K and 38K band 3 polypeptides (Jennings & Passow, 1979). These [$^3H_2$]DIDS cross-linked cells were reequilibrated with 5PBS7.4 and EMA labeled as described above. All erythrocyte samples were hemolyzed by dilution into 5P8.0 and washed free of hemoglobin. The labeled ghosts were dissolved in 5P8.0 containing SDS and DTT (final concentrations of 1% and 1 mM, respectively), and aliquots were taken for [$^3H_2$]DIDS quantitation by liquid scintillation counting and EMA quantitation by spectrophotometry. The dissolved ghosts were stored frozen at −70 °C until further analysis by SDS–PAGE. The 8% T gels employed the buffer system of Laemmli (1970), but the cross-linking reagent DADT (0.32%, w/v) was used instead of bis(acrylamide) to

[2] The following convention is used throughout this report to simplify the presentation of double labeling experiments: BSSDA/EMA, BSSDA prelabeling followed by EMA labeling; EMA/BSSDA, EMA prelabeling followed by BSSDA labeling; [$^3H_2$]DIDS/EMA, [$^3H_2$]DIDS labeling followed by EMA labeling; EMA/[$^3H_2$]DIDS, EMA prelabeling followed by [$^3H_2$]DIDS labeling.

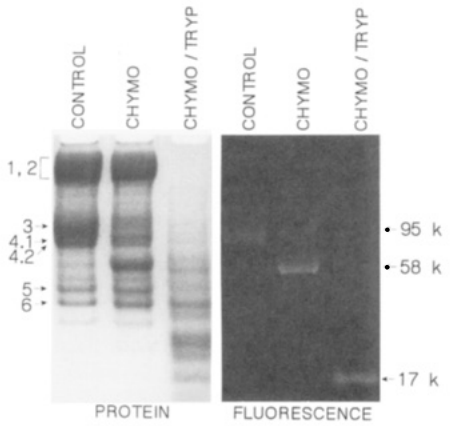

FIGURE 1: SDS–PAGE analysis of EMA-labeled erythrocyte ghosts. Intact erythrocytes were labeled with EMA as described under Experimental Procedures. Labeled cells were treated with chymotrypsin and hemolyzed, and the ghost membranes were washed free of hemoglobin and treated with trypsin. The ghosts were dissolved in SDS and DTT and electrophoresed on a 3–12% T gradient SDS gel employing the buffer system of Fairbanks et al. (1971). Eosin was detected by exposing the gel to UV light (right panel) followed by visualization of the protein bands by Coomassie blue staining (left panel). Protein bands are labeled on the left according to Fairbanks et al. (1971). The band labeled 58K corresponds to the M_r 58 000 band 3 polypeptide produced by extracellular chymotrypsin cleavage, and the band labeled 17K corresponds to the M_r ~17 000 band 3 fragment produced by extracellular chymotrypsin and intracellular trypsin cleavage.

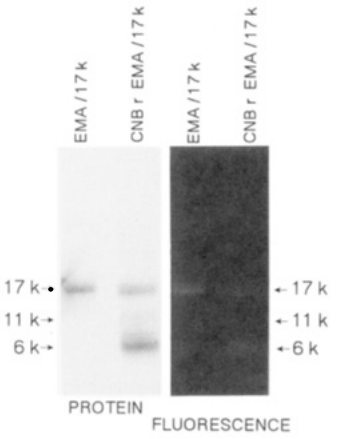

FIGURE 2: SDS–PAGE separation of CNBr-cleaved EMA/17K peptide. Electrophotoretically purified EMA/17K was treated with CNBr as described under Experimental Procedures, followed by lyophilization. The dried peptide mixture was dissolved in electrophoresis sample buffer and electrophoresed on a 15–20% T gradient SDS gel employing the buffer system of Laemmli (1970). Eosin was detected by exposing the gel to UV light (right panel) followed by visualization of the peptides by silver staining (left panel). Under these cleavage conditions, EMA/17K was cleaved primarily to C-terminal M_r 11K (which does not stain intensely) and N-terminal M_r 6K peptides. The eosin fluorescence was clearly found in the 6K peptide band.

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    361              365                   370                374
A: Gly-Leu-Asp-Leu-Asn-Gly-Gly-Pro-Asp-Asp-Pro-Leu-Gln-Gln
B: Gly-Leu-Asp-Leu- X -Gly-Gly-Pro-Asp-Asp-Pro-Leu-

    422          425                   430                435
C: Thr-Phe-Gly-Gly-Leu-Leu-Gly-Glu-Lys-Thr-Arg-Asn-Gln-Met
D:          Gly-Gly-Leu-Leu-Gly-Glu- X -Thr-Arg-Asn-Gln-
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FIGURE 3: Partial amino acid sequences of EMA-labeled 6K CNBr peptide and chymotrypsin-treated EMA/17K peptide. The amino acid sequence of the purified EMA-labeled 6K peptide (B) derived from CNBr cleavage of the EMA/17K peptide is aligned with the N-terminal sequence of the 17K peptide [(A) Tanner et al., 1988; Lux et al., 1989]. No single PTH-amino acid could be conclusively identified in the fifth sequencing cycle. The sequence of the RP-HPLC-purified EMA-labeled *chymotryptic* peptide (D) is aligned with a segment (amino acids 422–435) of the 17K transmembrane segment of band 3 [(C) Tanner et al., 1988; Lux et al., 1989]. This was the only region of total sequence homology between the EMA-labeled peptide and the full length of the band 3 sequence. The amino acid derivative peak in the seventh cycle, which corresponds to Lys-430, did not correlate with any of the PTH-amino acid standards.

allow subsequent solubilization of the gel (Young et al., 1980). This substitution did not affect the electrophoretic separation of the membrane proteins. The EMA label was detected by excitation by UV light, protein bands were visualized by Coomassie blue R-250 staining, and the radioactivity was quantitated by scintillation counting after slicing the stained gel, dissolving the gel slices with 2% periodic acid in scintillation vials, and addition of aqueous scintillation fluid.

EPR Measurements. X-band EPR spectra were recorded on a Bruker ESP-300 spectrometer equipped with an ER-4103 TM_{110} cavity and an ER-4111VT variable-temperature unit. Samples were contained in a WG-812 (Wilmad Glass Co.) aqueous flat cell for recording all EPR spectra. Spectra of BSSDA-labeled ghosts were recorded at 20 °C with the following instrument settings: 9.80-GHz microwave frequency, 10-mW microwave power, 100-kHz modulation frequency, 1.0-G modulation amplitude (peroxylaminedisulfonate calibrated; Beth et al., 1983), ±50-G scan range, 1024 data points per spectrum. Spectra of the HPLC fractions were recorded with the same instrument settings except the microwave power was set to 20 mW and the modulation amplitude was 3.2 G.

RESULTS

Localization of the EMA Reaction Site. Macara et al. (1983) showed that EMA labels band 3 in the M_r 17K integral membrane peptide generated by sequential extracellular chymotrypsin and intracellular trypsin cleavages. We have confirmed this earlier observation as shown in Figure 1. The 17K peptide has one methionine residue at position 435 which in previous work (Jennings & Nicknish, 1984) has been shown to provide a convenient CNBr cleavage site. Cyanogen bromide cleavage at Met-435 results in the generation of two peptides, the N-terminal one having an apparent molecular weight of approximately 6K, and the C-terminal one, which does not stain as intensely, having an apparent molecular weight of approximately 11K (Jennings & Nicknish, 1984). Under very harsh cleavage conditions, the 11K peptide can also be cleaved at a non-methionine peptide bond (Ramjeesingh & Rothstein, 1982). The CNBr cleavage conditions we have employed result in primarily a single CNBr cleavage at Met-435 and have allowed us to localize the eosin reaction site within the 17K peptide. After reaction of EMA/17K with CNBr, the resulting peptides were separated by SDS–PAGE. The eosin fluorescence was clearly detected in the 6K CNBr peptide as shown in Figure 2. This band was electroeluted, concentrated by lyophilization, and sequenced through 10 cycles. The amino-terminal sequence of the EMA-labeled peptide was the same as the amino-terminal sequence of the intact 17K peptide (Figure 3), indicating that the amino acid labeled by EMA was located between Gly-361 and Met-435.

Sequencing of the Eosin-Labeled Peptide. In order to define the amino acid modified by EMA, purified EMA/17K peptide was extensively digested with chymotrypsin as described under Experimental Procedures, and the resulting peptide mixture was separated by RP-HPLC. The amino acid sequence of the major eosin-containing peptide (85–90% of the eosin eluted

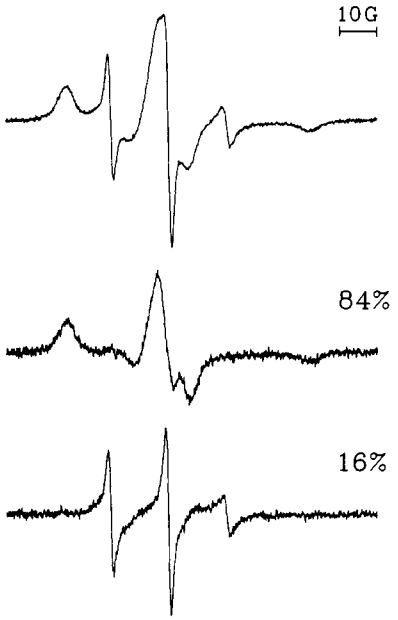

FIGURE 4: Deconvolution of the EPR spectrum of BSSDA/EMA erythrocyte ghosts. Ghosts were made from erythrocytes labeled with 50 μM BSSDA followed by EMA labeling as described under Experimental Procedures. The two motional components of the composite (upper) spectrum were digitially separated and numerically integrated in order to quantitate the contributions of the slow (84%, center) and fast (16%, lower) components to the composite spectrum.

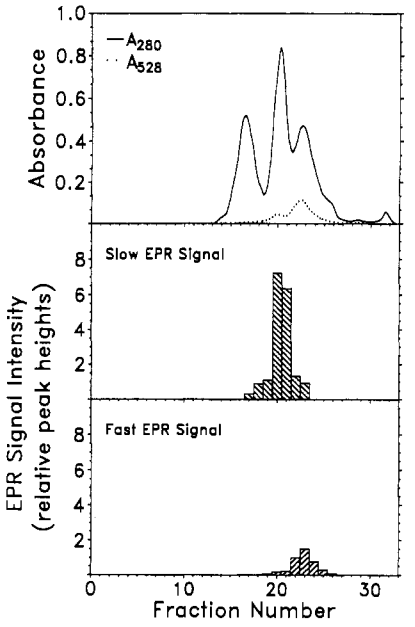

FIGURE 5: HPLC TSK-4000 separation of BSSDA/EMA erythrocyte ghost membrane proteins. Chromatographic conditions were the same as described by Anjaneyulu et al. (1989) except 3.5 mg of membrane protein was injected onto the column and the flow rate was 0.8 mL/min. The absorbances at 280 (solid line) and 528 nm (dotted line) were monitored, 1.0-min duration fractions were collected, and EPR spectra were recorded on the fractions. The fast- and slow-motion components in those fractions containing both EPR signals were separated by digital subtraction, and EPR signal intensity was quantitated by measurement of the heights of the center EPR peaks.

from the column) is presented in Figure 3. The average repetitive yield for this peptide through 11 sequencing cycles was 79%. The N-terminal six amino acids detected align perfectly with residues Gly-424 through Glu-429 of the human sequence. Therefore, this peptide resulted from chymotrypsin cleavage of EMA/17K at Phe-423. At cycle seven, the PTH-amino acid detected did not correspond with any of the PTH-amino acid standards. This is the cycle at which Lys-430 should have appeared. Cycles 8 through 11 yielded amino acids which aligned perfectly with Thr-431 through Gln-434 of the human sequence. These data, taken with the observation that the EMA-labeled amino acid was contained in the N-terminal 6K CNBr peptide which terminates at Met-435, strongly suggest that Lys-430 is the covalent reaction site. It is noteworthy that there are no other lysyl residues and no cysteinyl residues in the 6K CNBr peptide.

Assignment of Lys-430 as the EMA reaction site is further supported by additional sequence determinations which were carried out on the EMA/17K peptide which was extensively digested with thermolysin or with papain (Cobb & Beth, 1988). These two protease treatments yielded very short eosin-containing peptides which after purification by RP-HPLC yielded the sequences Leu-Gly-Glu-X or Glu-X-Thr-Arg, respectively (data not shown).

Labeling of Erythrocytes with EMA and BSSDA. Since BSSDA and EMA have both been employed in spectroscopic studies of band 3 structure and/or dynamics, it is important to investigate if they react with band 3 at overlapping loci or if their reaction sites are spatially separate. We have investigated the effect of prelabeling erythrocytes with one probe on subsequent labeling by the other probe. As shown in previous work, labeling of intact erythrocytes with the bifunctional nitroxide spin-probe BSSDA yields a two-component EPR specltrum, the major component (75–85% of the total EPR signal) being spin-labeled band 3 and the minor component being spin-labeled membrane lipid (Beth et al., 1986; Anjaneyulu et al., 1989). These motional components can be separated by digital subtraction and numerically integrated in order to quantitate the contribution of each to the composite spectrum.

When BSSDA-labeled erythrocytes were then labeled with EMA employing the standard labeling conditions described under Experimental Procedures, the line shape of the resulting EPR spectrum (Figure 4, upper spectrum) is the same as found in BSSDA-labeled control cells (Anjaneyulu et al., 1989). The slow-motion spin-labeled band 3 component (Figure 4, center spectrum) contains 84% of the total EPR signal in this experiment, and the fast-motion spin-labeled lipid component (Figure 4, lower spectrum) comprises the remaining 16%. Quantitation of the eosin fluorescence in ghost membranes prepared from these doubly labeled *intact* cells indicated that the EMA labeling was inhibited by approximately 80% relative to erythrocytes labeled with EMA alone. It is important to note that the BSSDA labeling protocol employed results in 0.9–1.0 BSSDA molecule reacted per band 3 monomer present based upon spin concentration in the slow-motion signal (Beth et al., 1986) and that the cross-linked product formed spans the extracellular chymotrypsin cleavage site of greater than 85% of the band 3 monomers present (Anjaneyulu et al., 1989).

The column profile of membrane proteins from these BSSDA/EMA-labeled erythrocytes separated by size exclusion on a HPLC TSK-4000 column is shown in Figure 5. Absorbance monitoring at 280 and 528 nm indicated that only approximately 25% of the eosin comigrated with the second large protein peak, which is shown by SDS–PAGE analysis of the column fractions to consist primarily of band 3 (Figure 6; fractions 20 and 21). The remainder of the eosin in these membranes is found in several lower molecular weight proteins (Figure 6; fractions 22–25). In contrast, when EMA-prelabeled membrane proteins were separated on the HPLC TSK-4000 column, virtually all of the eosin comigrated with the large band 3 peak, and there was no eosin absorbance peak

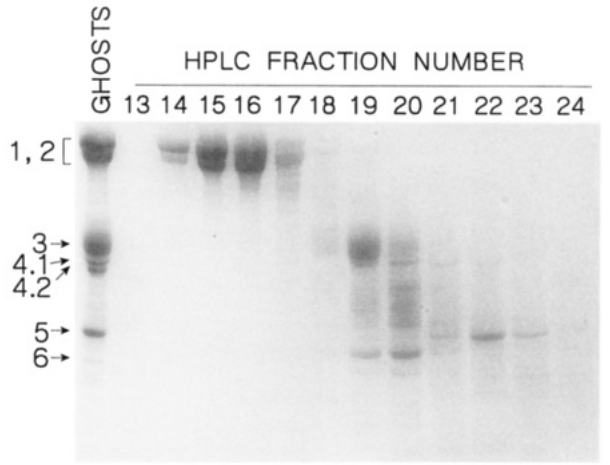

FIGURE 6: SDS–PAGE separation of BSSDA/EMA erythrocyte ghost HPLC TSK-4000 fractions. Sixty-microliter aliquots of fractions 13–24 from the HPLC fractionation shown in Figure 5 were electrophoresed in a 5–12% T SDS gel (Laemmli, 1970). A sample of the ghosts (75 μg) before HPLC was run in the left lane; protein bands are labeled according to Fairbanks et al. (1971). The protein bands were visualized by staining with Coomassie blue. This gel confirms that the two fractions in which most of the slow-motion EPR signal was found (Figure 5, fractions 20 and 21) contain band 3 as the predominant protein.

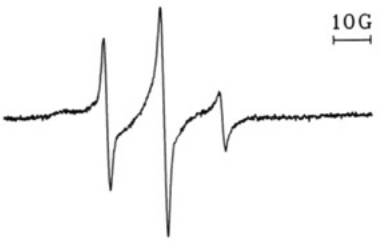

FIGURE 7: EPR spectrum of EMA/BSSDA erythrocyte ghosts. Ghosts were prepared from erythrocytes labeled with EMA followed by BSSDA labeling as described under Experimental Procedures. After spectral subtraction and quantitation, it was found that the quantity of spin-label in the fast-motion component is the same as in BSSDA/EMA membranes (Figure 4), and in BSSDA-labeled control membranes (not shown). The amplitude of the slow-motion component of the EMA-prelabeled membranes was reduced by 90% compared to the BSSDA prelabeled cells (Figure 4).

in the fraction 22–25 region (data not shown). Therefore, BSSDA actually inhibited EMA labeling of band 3 by approximately 95% but appeared to cause a slight enhancement of labeling of other, smaller membrane proteins by EMA. The fast- and slow-motion EPR signals were partially resolved, with the slow-motion signal being present in the HPLC fractions containing band 3 (Figure 6; fractions 20 and 21) and the fast-motion signal eluting from the column later (Figure 6; fractions 22–24), as reported by Anjaneyulu et al. (1989).

Prelabeling of erythrocytes with EMA under the standard labeling conditions (see Experimental Procedures), which results in an approximately 1:1 EMA to band 3 labeling stoichiometry, caused a 90% decrease in the amplitude of the slow-motion component relative to the BSSDA control cells, whereas the magnitude of the fast-motion component was essentially unchanged (Figure 7). These data show that each of these spectroscopic probes greatly diminishes labeling of band 3 by the second probe added, suggesting that they are competing for an overlapping reaction site.

Labeling of Intact Erythrocytes with EMA and [$^3H_2$]DIDS. Treatment of *intact* erythrocytes with chymotrypsin cleaves band 3 into M_r 58K and M_r 38K polypeptides. Incubation of erythrocytes with [$^3H_2$]DIDS in 5PBS at pH 7.4 results in labeling of the 58K band 3 polypeptide and some of the

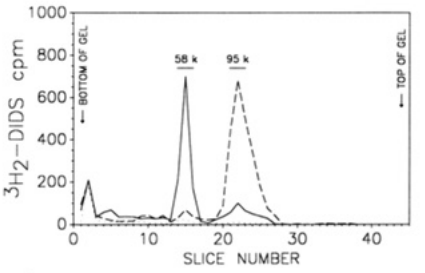

FIGURE 8: [$^3H_2$]DIDS quantitiation of chymotrypsin-treated [$^3H_2$]DIDS/EMA and EMA/[$^3H_2$]DIDS erythrocyte ghost membrane proteins. Intact erythrocytes were treated with chymotrypsin followed by labeling with [$^3H_2$]DIDS in 5PBS7.4 as described under Experimental Procedures. The [$^3H_2$]DIDS-labeled cells were then either labeled with EMA or equilibrated with 150 mM $NaHCO_3$ (pH 9.5) and incubated at 37 °C to allow cross-linking of the band 3 chymotryptic polypeptides followed by labeling with EMA in 5PBS7.4. Alternatively, chymotrypsin-treated erythrocytes were labeled with EMA as described under Experimental Procedures. The cells were then labeled with [$^3H_2$]DIDS in 5PBS7.4. Ghost membranes from these cells were electrophoresed (150 μg of protein/lane), and the [$^3H_2$]DIDS radioactivity in the gel was quantitated as described under Experimental Procedures. The [$^3H_2$]DIDS radioactivity was found primarily in the M_r 58K N-terminal polypeptide of band 3 when cells were labeled in 5PBS7.4 (solid line), and subsequent incubation in alkaline buffer facilitated cross-linking of the 58K and 38K polypeptides (dashed line). Prelabeling with EMA prevented the subsequent covalent reaction of [$^3H_2$]DIDS with the 58K or 38K polypeptides (dotted line). The small peak near the bottom of the gel is assumed to result from [$^3H_2$]DIDS labeling of membrane lipids.

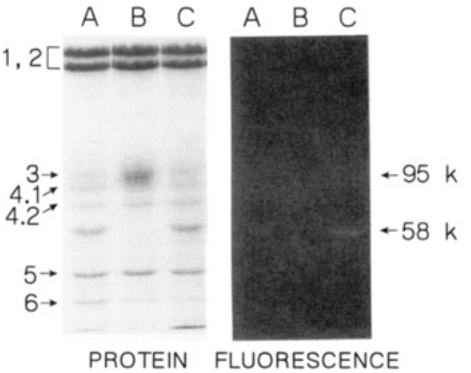

FIGURE 9: SDS–PAGE of chymotrypsin-treated [$^3H_2$]DIDS/EMA and EMA/[$^3H_2$]DIDS erythrocyte ghost membrane proteins. Labeled ghost membranes were prepared and electrophoresed as described in the legend to Figure 8 and under Experimental Procedures. Eosin fluorescence (right panel) was detected by exposing the gel to UV light, followed by visualization of the protein bands by Coomassie blue staining (left panel). [$^3H_2$]DIDS prelabeling reduced subsequent EMA labeling of band 3 by over 90% in both non-cross-linked [[$^3H_2$]DIDS (PBS)/EMA, lane A], and cross-linked [[$^3H_2$]DIDS (PBS/$NaHCO_3$)/EMA, lane B] erythrocyte membranes. The eosin fluorescence was found in the 58K polypeptide in chymotrypsin-treated EMA-prelabeled membranes [EMA/[$^3H_2$]DIDS (PBS), lane C].

membrane lipids. After the unreacted [$^3H_2$]DIDS was washed away, incubation of the cells at 37 °C for 1 h in 150 mM bicarbonate, pH 9.5, results in [$^3H_2$]DIDS cross-linking of the M_r 58K and 38K polypeptides (Jennings & Passow, 1979). Formation of the cross-linked product can be confirmed by the reappearance of the M_r 95K band and the disappearance of the M_r 58K band on SDS–PAGE, which is also reflected by the concomitant transfer of [$^3H_2$]DIDS radioactivity to the M_r 95K band (Figure 8).

Prelabeling of nontreated (data not shown) or chymotrypsin-treated erythrocytes with [$^3H_2$]DIDS at pH 7.4 decreases EMA labeling of the same cells by over 90%. Analysis of the chymotrypsin-treated cells indicates that the [$^3H_2$]DIDS radioactivity was found on the 58K polypeptide (Figure 8, solid line, and Figure 9). Cross-linking of the [$^3H_2$]DIDS-labeled

erythrocytes by incubation at pH 9.5 did not change the amount of inhibition of subsequent EMA labeling (Figure 9). Prelabeling erythrocytes with EMA decreased [$^3H_2$]DIDS labeling of band 3 at pH 7.4 by approximately 95%, but did not reduce [$^3H_2$]DIDS labeling of membrane lipids (Figure 8, dotted line). EMA prelabeling also essentially totally blocked [$^3H_2$]DIDS labeling of the 38K band 3 polypeptide when the [$^3H_2$]DIDS incubation occurred in the bicarbonate buffer at pH 9.5 (data not shown). These labeling studies suggest that EMA and [$^3H_2$]DIDS also compete for an overlapping reaction site.

Discussion

Identification of Lys-430 as the primary band 3 reaction site for EMA in the human erythrocyte has provided information on the arrangement of this protein in the membrane. At pH 7.4, EMA is negative charged and apparently does not permeate the cell membrane to an appreciable extent as evidenced by the absence of any detectable labeling of known cytoplasmic or cytosolic proteins in the erythrocyte. Facile labeling of Lys-430 by EMA in *intact* cells strongly suggests that this residue is readily accessible from the extracellular space.

Extracellular orientation of Lys-430 (Lys-449 in the murine sequence; Kopito & Lodish, 1985) is supported by working structural models for human band 3 based upon its primary structure (Tanner et al., 1988; Lux et al., 1989). There is general agreement that the amino-terminal ~400 amino acids of human band 3 form an elongated water-soluble peptide which extends into the cytoplasm of the cell [see Low (1986)]. However, starting at Ala-400, there are 29 consecutive uncharged amino acids terminating with Gly-428, of which Gln-404 to Gly-424 are predicted, based upon hydropathy calculations, to form a membrane-spanning domain (Lux et al., 1989). Such a membrane-spanning domain would result in Lys-430 being at, or near, the extracellular membrane–water interface and therefore available for chemical modification by membrane-impermeant reagents. Jennings and Nicknish (1984) showed that Lys-430 could be reductively methylated in *intact* cells under conditions that favor labeling of extracellular, but not intracellular, lysines. Thus, previous work (Jennings & Nicknish, 1984) and the present work support the model of Lux et al. (1989) suggesting that residues Ala-400 through Gly-428 form a membrane-spanning domain.

Macara et al. (1983) carried out proteolysis of band 3 to show that the integral membrane 17K peptide was specifically labeled with EMA when labeling was carried out on *intact* cells, an observation which we have confirmed (Figure 1). This peptide contains four lysine residues (Lys-430, Lys-539, Lys-542, and Lys-551) and one cysteine residue (Cys-479), the latter being an attractive potential candidate for reaction with the maleimide function of EMA (Tanner et al., 1988). Previous studies have shown that Cys-479 is not reactive with *N*-ethylmaleimide in *intact* cells (Rao, 1979; Rao & Reithmeier, 1979; Ramjeesingh et al., 1983), leading to its characterization as "cryptic". As with *N*-ethylmaleimide, we have found no evidence for Cys-479 reaction with EMA in the present studies. Cleavage of EMA/17K with CNBr showed that essentially all of the eosin fluorescence was recovered in the 6K N-terminal peptide (Figure 2) which rules out any significant reaction with Cys-479 (or with Lys-539, Lys-542, and Lys-551). It is not clear why this residue does not react with EMA since cysteine is normally a preferred target for maleimide reagents. However, since EMA acts as an affinity label for band 3, it is possible that positioning of the maleimide moiety through specific interactions between eosin and band 3 enhances reaction with Lys-430 due to local concentration effects.

It is important, particularly for spectroscopic applications employing EMA as an exogenous probe, to estimate the fraction of labeling which occurs at Lys-430 versus other sites on band 3. By assuming a value of 25 ± 5% (by weight) of the ghost membrane protein being band 3 (Fairbanks et al., 1971), a stoichiometry of 1.0 ± 0.2 (n = 15) EMA molecules per band 3 subunit present can be calculated on the basis of this quantitation of band 3 and an extinction coefficient at 530 nm for eosin bound to protein of 96 mM^{-1} cm^{-1} (Tsuji et al., 1988). This suggests that there is a single reaction site for EMA on each band 3 monomer under the reaction conditions employed in this study, but does not directly rule out two or more reaction sites which have partial reactivity with EMA that fortuitously results in an overall stoichiometry of 1 EMA per band 3 monomer. It is evident from the in situ proteolytic cleavages of band 3 that essentially all of the eosin fluorescence is retained in the 17K integral membrane peptide (Figure 1). As discussed in the previous paragraph, all of the experimental evidence we have obtained and presented supports Lys-430 as the only detectable amino acid residue modified by EMA. Therefore, we conclude that the vast majority of the EMA present in labeled erythrocyte membranes is covalently bound to Lys-430.

From a mechanistic standpoint, it is of interest to determine which amino acids, and particularly which positively charged amino acids, are crucial for anion-exchange activity. Nigg and Cherry (1979) showed that EMA labeling of band 3 stoichiometrically inhibited SO_4^{2-} exchange in erythrocytes. However, other studies have indicated that Lys-430, which we have identified as the covalent reaction site for EMA, may not be a crucial residue for anion exchange (Jennings & Nicknish, 1984). It may be that the eosin ring system with its anionic groups projects into the anion binding site of band 3 and thereby inhibits exchange via interactions with residues which are crucial for function. This interpretation is consistent with the energy-transfer measurements of Macara et al. (1983) where it was shown that quenching of EMA fluorescence by Cs^+ was greatest from the cytoplasmic side of the membrane. Though EMA reacts with Lys-430 which is predicted to be at the extracellular surface, its substantial length coupled with the length of the lysine side chain may allow the xanthine moiety to interact with a region of the anion binding site at considerable depth in the membrane. This model for inhibition by EMA is also consistent with a previous study which provided data indicating that the extracellularly accessible anion binding site of band 3 is located in a cleft which extends into the membrane (Rao et al., 1979).

Results from the present studies have shown that EMA, BSSDA, and [$^3H_2$]DIDS all interfere with each other for reaction with band 3 in sequential labeling experiments. EMA essentially blocks reaction of BSSDA with band 3, and BSSDA essentially blocks subsequent reaction of EMA (Figures 4–7). These results suggest that the spectroscopic probes EMA and BSSDA are monitoring a common region of band 3 which is in, or near, the functionally important extracellular anion binding site.

Previous work by Macara et al. (1983) indicated that prelabeling of erythrocytes with DIDS blocked subsequent reaction of EMA by 85%. We have confirmed this result using [$^3H_2$]DIDS as shown in Figures 8 and 9. Moreover, we have shown that EMA essentially blocks covalent reaction of [$^3H_2$]DIDS with band 3 (Figures 8 and 9). This mutual exclusion must occur at the level of competition for spatial

access to a region of the anion binding site since the covalent reaction of [$^3H_2$]DIDS to band 3 involves an amino acid residue on 17K which is distinct from Lys-430 [see Passow (1986)] but as yet not uniquely defined (Garcia & Lodish, 1989). It is also noteworthy that EMA blocks the covalent reaction of [$^3H_2$]DIDS with its other potential reaction site on the 38K integral membrane polypeptide. Two possible reasons for this inhibition include (a) EMA binding and/or reaction with band 3 induces a conformational change, such as a partial transport event, which does not permit the isothiocyano group of [$^3H_2$]DIDS access to the lysine residue on 38K or (b) the negatively charged eosin portion of EMA stearically or electrostatically inhibits [$^3H_2$]DIDS binding to band 3 in its normal configuration such that covalent attachment cannot occur.

In summary, we have provided evidence that Lys-430 is the major band 3 reaction site for EMA in *intact* human erythrocytes. When EMA has covalently reacted with band 3 at this site, reaction of BSSDA or [$^3H_2$]DIDS with band 3 is effectively blocked, suggesting that these three chemically diverse protein modification reagents are competing for either a common reaction site or reaction sites which spatially overlap. Sequential labeling studies where EMA is the second reagent added fully support this conclusion. The data presented in these studies, taken with those from identification of additional probe reaction sites on band 3 which undoubtedly will follow now that the primary structure of the human erythrocyte protein is known, should aid in construction of improved models for the structure of band 3 in the membrane and for understanding the mechanisms whereby various inhibitors are able to block anion exchange.

ACKNOWLEDGMENTS

We thank Dr. Thomas J. Lukas and Dr. D. Martin Watterson (Howard Hughes Medical Institute and Department of Pharmacology, Vanderbilt University) for assistance with peptide sequencing, Dr. K. Balasubramanian (Department of Molecular Physiology and Biophysics, Vanderbilt University) for assistance with the EPR spectral data display, and Dr. J. V. Staros (Department of Biochemistry, Vanderbilt University) for review of the manuscript.

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