Enzymatic Methylation of Band 3 Anion Transporter in Intact Human Erythrocytes†

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ABSTRACT: Band 3, the anion transport protein of erythrocyte membranes, is a major methyl-accepting substrate of the intracellular erythrocyte protein carboxyl methyltransferase (*S*-adenosyl-L-methionine: protein-D-aspartate *O*-methyltransferase; EC 2.1.1.77) [Freitag, C., & Clarke, S. (1981) *J. Biol. Chem. 256*, 6102–6108]. The localization of methylation sites in intact cells by analysis of proteolytic fragments indicated that sites were present in the cytoplasmic N-terminal domain as well as the membranous C-terminal portion of the polypeptide. The amino acid residues that serve as carboxyl methylation sites of the erythrocyte anion transporter were also investigated. ^{3}H-Methylated band 3 was purified from intact erythrocytes incubated with L-[*methyl*-^{3}H]methionine and from trypsinized and lysed erythrocytes incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. After proteolytic digestion with carboxypeptidase Y, D-aspartic acid β-[^{3}H]methyl ester was isolated in low yields (9% and 1%, respectively) from each preparation. The bulk of the radioactivity was recovered as [^{3}H]methanol, and the amino acid residue(s) originally associated with these methyl groups could not be determined. No L-aspartic acid β-[^{3}H]methyl ester or glutamyl γ-[^{3}H]methyl ester was detected. The formation of D-aspartic acid β-[^{3}H]methyl esters in this protein in intact cells resulted from protein carboxyl methyltransferase activity since it was inhibited by adenosine and homocysteine thiolactone, which increases the intracellular concentration of the potent product inhibitor *S*-adenosylhomocysteine, and cycloleucine, which prevents the formation of the substrate *S*-adenosyl-L-[*methyl*-^{3}H]methionine.

Protein carboxyl methyltransferases are widely distributed in nature and catalyze the formation of protein methyl esters. For example, a specific bacterial methyltransferase is responsible for the methylation of several membrane chemoreceptors on L-glutamyl residues, and these reactions appear to regulate the sensory output of these proteins (Stock et al., 1985a,b). Protein carboxyl methyltransferases are also present in many mammalian tissues. The physiological roles of these eucaryotic enzymes are poorly understood, although it has been hypothesized that these reactions can also regulate the function of the various methyl-accepting substrates (Gagnon & Heisler, 1979; O'Dea et al., 1981).

We have studied eucaryotic protein carboxyl methylation in human erythrocytes [for a review, see Clarke (1985)]. Several major methylated proteins have been identified in the membrane fraction of intact cells including the cytoskeletal proteins band 2.1 and band 4.1, as well as band 3, the anion transport protein (Freitag & Clarke, 1981). A methylated residue has been isolated in low yield from proteolytic digests of unfractionated methylated erythrocyte membranes and has been identified as the unnatural D isomer of aspartic acid β-methyl ester (McFadden & Clarke, 1982; Clarke et al., 1984). Studies using synthetic peptides as methyl acceptors have indicated that a second methylated product is L-aspartic acid α-methyl ester (Murray & Clarke, 1984; Aswad, 1984). The origin of the substrates for these reactions (L-isoaspartyl and presumably D-aspartyl residues) in cells is uncertain, although they may arise as a consequence of spontaneous racemization or deamidation events. On the basis of these data, it appears that the methylation reaction may be involved in the metabolism of age-modified proteins.

In the present work, we have focused on delineating the sites of methylation of the band 3 anion transport protein in human erythrocytes. The structure and function of this protein have been reviewed recently (Macara & Cantley, 1983; Jay & Cantley, 1986). Band 3 is a major endogenous substrate for the human erythrocyte protein carboxyl methyltransferase in both intact (Freitag & Clarke, 1981) and broken cell preparations (Terwilliger & Clarke, 1981). On the basis of inhibitor studies, this methylation reaction does not appear to directly regulate the anion transport function of this protein (Lou & Clarke, 1986). Band 3 is an ideal candidate for these experiments because previous work has established the topography of this protein within the bilayer [see, for example, Jennings & Nicknish (1984)] and functional domains have been identified for anion transport (Grinstein et al., 1978; Ramjeesingh et al., 1980), cytoskeletal association (Bennett & Stenbuck, 1979; Bennett, 1982), and binding of cytosolic proteins (Murthy et al., 1981). Partial sequence information is available for the human band 3 protein (Mawby & Findlay, 1982; Brock et al., 1983; Kaul et al., 1983), and a complete sequence of the mouse protein has recently been proposed (Kopito & Lodish, 1985).

In this paper, we report on the identification of a methylated residue and the localization of the major methylation sites in band 3 of membranes isolated from erythrocytes incubated

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with L-[*methyl*-^{3}H]methionine. These probably represent the endogenous sites, since cells were incubated under conditions of physiological pH, temperature, and methionine concentration.

Experimental Procedures

Materials. L-[*methyl*-^{3}H]Methionine and *S*-adenosyl-L-[*methyl*-^{3}H]methionine were purchased from Amersham. Adenosine, L-homocysteine thiolactone, cycloleucine, L-methionine, *S*-adenosyl-L-homocysteine, phenylmethanesulfonyl fluoride, penicillin G, streptomycin sulfate, and all proteases were purchased from Sigma. L-Aspartic acid β-methyl ester hydrochloride was purchased from Vega Biochemicals. D-Aspartic acid β-methyl ester hydrochloride and L-leucine *N*-carboxyanhydride were prepared as described (McFadden & Clarke, 1982). Reagents for gel electrophoresis were obtained from Bio-Rad. Reagents and buffers for amino acid analysis were obtained from Pierce. All other reagents were analytical reagent grade.

Preparation of Erythrocytes. Human erythrocytes were prepared from freshly drawn blood as described (Barber & Clarke, 1984).

Carboxyl Methylation of Erythrocyte Membrane Proteins. Intact cells were incubated under the following conditions. An aliquot of L-[*methyl*-^{3}H]methionine (72–93 Ci/mmol, 13.9–10.8 μM, 3.5 volumes) was lyophilized to dryness in a polycarbonate centrifuge tube. It was resuspended in 2.5 volumes of isotonic phosphate buffer (118 mM sodium phosphate, pH 7.4) containing 16.7 mM glucose prior to the addition of 1 volume of washed erythrocytes. Incubation was carried out at 37 °C with shaking for 3 h (28.6% hematocrit, final concentration of 10.8–13.9 μM L-[*methyl*-^{3}H]-methionine). For methylation-inhibited samples, either 5 mM each of adenosine and homocysteine thiolactone or 50 mM cycloleucine was included in the incubation buffer. At the end of the incubation period, cells were diluted with at least 30 volumes of ice-cold 118 mM sodium phosphate, pH 7.4, and then collected by centrifugation. Pelleted cells were hypotonically lysed by using at least 30 volumes of 5 mM sodium phosphate, pH 8 at 4 °C. Membranes were pelleted by centrifugation at 4 °C (26000*g*, 6 min). The supernatant and "button" underneath the soft membrane pellet were removed by aspiration. The membranes were washed in the same buffer at least twice more.

Band 3 was ^{3}H-methylated in lysed cells by the following procedure. Washed intact erythrocytes (450 μL) were fractured by freezing in a dry ice/2-propanol slurry (−70 °C) prior to addition of 37 μL of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (15 Ci/mmol, 66.7 μM). Lysed cells were allowed to incubate for 1 h at 37 °C and then resuspended in 30 volumes of ice-cold 5 mM sodium phosphate, pH 8. Membranes were washed as described above.

Separation of Extrinsic and Intrinsic Erythrocyte Membrane Proteins. Extrinsic membrane proteins including bands 1, 2, 2.1, 4.1, 5, and 6 and hemoglobin were removed from the intrinsic membrane proteins (mainly band 3 and the PAS bands) by extraction with 10% (v/v) acetic acid as described (Steck et al., 1976). The membrane pellet was resuspended in 10 volumes of 10% acetic acid and centrifuged at 27000*g* for 20–25 min at 4 °C. It was found that the extraction became more efficient when the suspension was allowed to incubate at room temperature for 10 min prior to centrifugation, and an additional wash with 100 volumes of 10% acetic acid was done. The resulting pellet, which contained the intrinsic proteins, was typically more tightly packed, reduced in volume, and transparent.

Proteolytic Fragmentation of Band 3: (*A*) *Intact Cells.* Trypsin and chymotrypsin digestions of erythrocyte external membrane polypeptides were performed according to procedures described by Drickamer (1976). Packed erythrocytes were incubated at 50% hematocrit in isotonic phosphate buffer with trypsin (from bovine pancreas, diphenylcarbamyl chloride treated, Sigma type XI, final concentration 19 μg/mL) for 3 h at 27 °C. To quench the protease digestion, phenylmethanesulfonyl fluoride in 95% ethanol was added to a concentration of 60 μg/mL. The sample was allowed to react for 30 min at 4 °C before cells were washed 3 or 4 times with 30–60 volumes of isotonic phosphate at 0 °C. Chymotrypsin digestions were carried out in phosphate-buffered saline at a 50% hematocrit. Washed erythrocytes were added to an equal volume of 2 mg/mL α-chymotrypsin (from bovine pancreas, tosyl-L-lysine chloromethyl ketone treated, Sigma type VII). The digestion was allowed to continue for 1–2 h at 24 °C. Phenylmethanesulfonyl fluoride was added to give a final concentration of 375 μg/mL, and the cells were processed as described above for the trypsin digestion. In all experiments, control cells were processed in parallel with the protease-treated cells, except that the proteases were omitted during the incubation. Carboxyl methylation of membrane proteins was carried out immediately following fragmentation.

(*B*) *Membranes.* Erythrocyte membranes were digested with trypsin after the procedure of Steck et al. (1978). Trypsin (40 μg/mL) in 5 mM sodium phosphate, pH 7.2, was added to an equal volume of membranes (4–6 mg of protein/mL). The digestion was allowed to continue for 1 h at 24 °C. The reaction was quenched by the addition of 10 volumes of 10% acetic acid, and membranes were extracted as described. Chymotrypsin digestion was performed as described by Ramjessingh et al. (1980) and Rao and Reithmeier (1979). Membranes were added to an equal volume of 5 mM sodium phosphate, pH 7.2, containing 0.1–4 mg of chymotrypsin/mL. The incubation was performed at 37 °C for 1 h, and the reactions were quenched as described above for the trypsin digestion.

Separation of Polypeptides by Sodium Dodecyl Sulfate/Urea–Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was performed in the pH 2.4 buffer system described by Fairbanks and Avruch (1972) and O'Connor and Clarke (1985) except that samples were denatured in buffer containing 4% sodium dodecyl sulfate (SDS)[1] and 4 M urea and the gels contained 6 M urea.

Quantitation of Methylation of Proteolytic Fragments Separated by Polyacrylamide Gel Electrophoresis. Protein methylation is measured by the radioactivity incorporated into specific polypeptide bands on SDS gels. Densitometric traces of the Coomassie-stained bands in wet gels and the film resulting from fluorography (O'Connor & Clarke, 1985) of the same processed gel were made by using a Helena Quick-Scan R & D instrument. The number of [^{3}H]methyl esters in polypeptide bands on gels was quantitated from the area of the densitometric trace of the film, and the number of polypeptides was calculated in a similar way from the densitometric trace of the Coomassie-stained gel and knowledge of the molecular weight of the protein species. Base lines for individual polypeptide bands were drawn in the same way for both the film and Coomassie profiles.

Isolation and Determination of Stereoconfiguration of Aspartic Acid β-[^{3}H]Methyl Esters from Band 3 Enriched

[1] Abbreviations: SDS, sodium dodecyl sulfate; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; L-LeuCA, L-leucine *N*-carboxyanhydride.

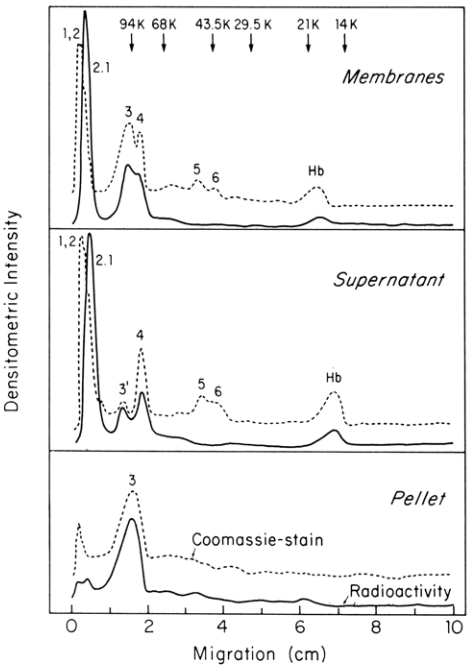

FIGURE 1: Separation of peripheral and integral membrane proteins by acetic acid extraction. Membranes were isolated from erythrocytes incubated with L-[*methyl*-^{3}H]methionine and extracted with 10% acetic acid as described under Experimental Procedures. The extracted supernatant and the residual pellet samples were analyzed by electrophoresis on a 7.5% polyacrylamide gel containing 1% SDS and 6 M urea. A densitometric trace of Coomassie blue stain (dashed line) is shown superimposed on a densitometric trace of a fluorograph of the same lane (solid line). The top panel represents 40 μg of total membrane protein before extraction (membranes from about 5.7 × 10^7 cells). The middle panel represents the supernatant, and the bottom panel represents the residual pellet after extraction of a similar amount of membranes. The migration positions of molecular weight standards are indicated by the arrows in the top panel. The band of residual hemoglobin is labeled Hb.

Membranes. Membranes (2–6 mg of protein/mL) prepared from trypsinized erythrocytes incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine under lysis conditions, and acetic acid extracted membranes from intact erythrocytes incubated with L-[*methyl*-^{3}H]methionine, were digested with equal volumes of carboxypeptidase Y (bakers' yeast, 2 mg of protein/mL, in pH 5 citrate buffer, Sigma) in the presence of 1% (v/v) Triton X-100 at 37 °C for 15–21 h. The digestion products were analyzed by cation-exchange and gel filtration chromatography as described in detail previously (Clarke et al., 1984). Stereoconfiguration of the radioactive material that coeluted with an added standard of aspartic acid β-methyl ester was determined by the diastereomeric dipeptide method of Manning and Moore (1968). The resulting L-leucyl dipeptides were separated by ion-exchange chromatography as described (McFadden & Clarke, 1982; Clarke et al., 1984).

Quantitation of Methyl Ester Formation in Band 3. Protein-associated methyl esters were defined as acid-precipitable, base-labile, alcohol/toluene-extractable, and volatile radioactivity. This is measured by the assay procedure described previously (O'Connor & Clarke, 1983). As assay performed with whole membranes provides a value of the picomoles of methyl ester in the membrane per milligram of total membrane protein. This value is multiplied by the percent of band 3 associated radioactivity relative to total protein-associated radioactivity to give the picomoles of methyl ester in band 3 per milligram of total membrane protein. The percent of radioactivity in whole membranes contributed by band 3 is defined as an area under the radioactive peak, in a densitometric trace of a fluorograph, comigrating with the band 3 Coomassie stain in the same lane divided by the area under all the radioactive peaks in the same densitometric trace.

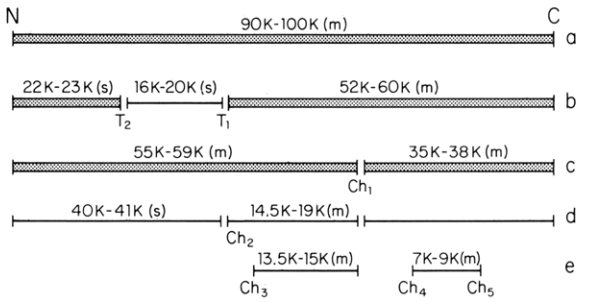

FIGURE 2: Major proteolytic cleavage sites of the band 3 polypeptide. N, N-terminal; C, C-terminal, K, ×10^3 daltons; Ch, chymotrypsin cleavage site; T, trypsin cleavage site. The suffixes s and m indicate soluble and membrane-bound fragments, respectively. Each cleavage site, as indicated by a breakage in the polypeptide, represents a small region of amino acid sequence that is sensitive to that particular protease. Stippled fragments represent methylated species. (a) No protease, or trypsin digestion of intact cells; (b) mild trypsin digestion of membranes; (c) chymotrypsin digestion of intact cells; (d) mild chymotrypsin digestion of membranes; (e) extensive chymotrypsin digestion of membranes.

RESULTS

Preparation of Isotopically Pure Band 3 by Acetic Acid Extraction of Intact Cell ^{3}H-Methylated Membranes. When intact erythrocytes are incubated with L-[*methyl*-^{3}H]-methionine, the major methylated species are bands 2.1, 3, and 4.1. The data in Figure 1 show that it is possible to extract the non-band 3 associated radioactivity from the membrane with acetic acid. The membrane pellet prepared in this fashion can then be used as a source of ^{3}H-methylated band 3.

Sites of Carboxyl Methylation on Band 3: Separation of the Cytoplasmic Domain and the Transmembrane Domain by Proteolytic Digestion. Band 3 is composed of two functionally and structurally dissimilar domains: a hydrophilic cytoplasmic portion (N-terminal) that associates with cytoskeletal and cytosolic proteins and a hydrophobic transmembrane portion (C-terminal) that transports anions across the bilayer. Mild trypsin digestion of unsealed membranes results in the release of the cytoplasmic domain of band 3, leaving the transmembrane domain in the membrane. The N-terminal-containing cytoplasmic domain consists of a water-soluble 41 000-dalton fragment, which is readily cleaved to yield two major polypeptides with molecular weights of 22 000–23 000 and 16 000–20 000 (Figure 2; Steck et al., 1978; Reithmeier, 1979). The membrane-bound fragment has an apparent molecular weight of 52 000, and it characteristically migrates as a diffuse band in a polyacrylamide gel (Ramjeesingh et al., 1980; Jennings et al., 1984).

When ^{3}H-methylated membranes were digested with trypsin and extracted with acetic acid as described above, the radioactivity and Coomassie stain of band 3 disappeared, and a new peak of radioactivity comigrated with a 60 000-dalton transmembrane fragment (Figure 3). This fragment was found to contain about 60% of the radioactive methyl groups associated with the intact band 3 polypeptide in a control sample incubated without protease. The rest of the methyl groups would be expected to be in the soluble fraction of the digest including the two major soluble fragments (16 000–20 000 and 22 000–23 000 daltons). The smaller fragment, which is sensitive to further digestion, is recovered invariably in low yield while the larger fragment can be obtained in good yield (Steck et al., 1976, 1978). Analysis of the supernatant after

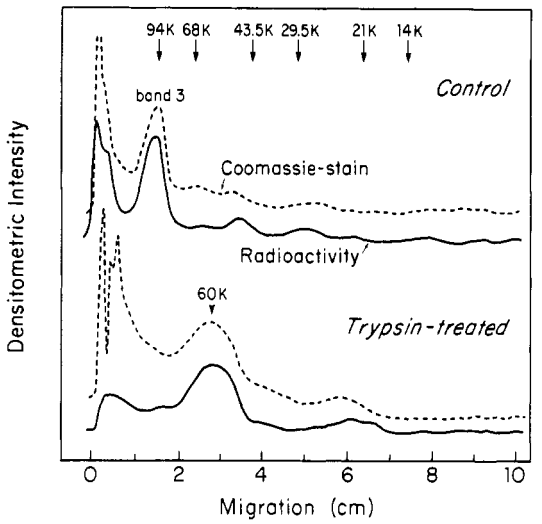

FIGURE 3: Removal of cytoplasmic domain of band 3 in erythrocyte membranes by trypsin digestion. Membranes isolated from ^{3}H-methylated intact cells were digested as described under Experimental Procedures. A control sample was incubated in parallel except that the protease was omitted. Membranes were extracted with acetic acid, and the residual pellets were analyzed by electrophoresis. The protein content and the corresponding radioactivity were detected and presented as described in the legend of Figure 1. In this particular experiment, the control lane represents a sample of 60 μg of total membrane protein (equivalent to 8.6×10^7 cells), and the trypsin lane represents a sample of digested membranes from an equivalent of 21×10^7 cells (2.4–2.5 times the amount in the control sample). The radioactivity recovered in the transmembrane trypsin fragment is 1.4 times the amount in the control sample. Therefore, after correction for the amount in the control sample materials applied according to the cell equivalence, the recovery of radioactivity in the proteolytic product was found to be 57% of that in the intact band 3 polypeptide (see Experimental Procedures for details). From the results of four separate experiments, the radioactivity of the 60 000-dalton transmembrane fragment was calculated to be 59% ± 3% of that of intact band 3 in an undigested sample. The value is expressed as mean ± standard deviation.

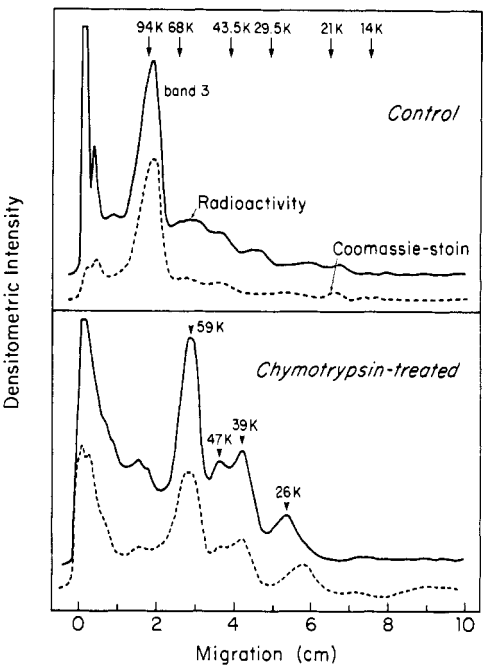

FIGURE 4: Fragmentation of band 3 by chymotrypsin digestion and separation of the major membrane polypeptides. Intact erythrocytes were treated with chymotrypsin and incubated with L-[*methyl*-^{3}H]-methionine as described under Experimental Procedures. Control cells were incubated in parallel without protease. Acetic acid extracted membranes were electrophoresed, and the data were analyzed as described in the legend of Figure 3. In this particular experiment, a sample of 80 μg of total membrane protein (equivalent to 11.4×10^7 cells) was applied onto the control lane. The chymotrypsin lane contained a sample prepared from 1.4 times the amount of material (16.0×10^7 cells) as in the control lane. The 59 000-dalton fragment was found to contain 68% of the radioactivity in the intact band 3 polypeptide after the amount of material applied to each gel lane had been normalized according to the cell equivalence. It was calculated from seven different experiments that 60% ± 9% of the radioactivity in an intact band 3 polypeptide can be recovered in the 59 000-dalton fragment after chymotrypsin digestion.

digestion revealed radioactivity in the 22 000-dalton N-terminal fragment (data not shown).

Proteolytic Fragmentation of Intact Erythrocytes Followed by Incubation with L-[*methyl*-^{3}*H*]*Methionine.* When intact erythrocytes are exposed to chymotrypsin, band 3 is cleaved into two major membrane-bound fragments with molecular weights of approximately 59 000 and 38 000 (Steck et al., 1978; Figure 2). The larger fragment contains the N-terminus and is easily detected in a polyacrylamide gel as a sharp Coomassie-staining band. The smaller fragment represents the C-terminal portion which migrates as a broad band in a polyacrylamide gel and stains poorly with Coomassie brilliant blue (Drickamer, 1976; Markowitz & Marchesi, 1981). Figure 4 shows that ^{3}H-methylated polypeptides migrating at 59 000 and 39 000 daltons were generated by chymotrypsin digestion of intact cells followed by incubation with L-[*methyl*-^{3}H]methionine. Similar results were obtained when erythrocytes were incubated with L-[*methyl*-^{3}H]methionine prior to chymotrypsin treatment. Approximately 60% of the radioactivity was found in the N-terminal 59 000-dalton fragment in each case. Taken together with the quantitation from the trypsin experiments, these results suggest that methylation sites occur in at least three regions of the band 3 polypeptide; about 40% of the methyl groups are located in the N-terminal 41 000-dalton fragment, 40% in the C-terminal 39 000-dalton fragment, and 20% in the central approximately 20 000-dalton region.

Localization of Methylation Site(s) in the Chymotrypsin 59 000-Dalton Transmembrane Fragment of Band 3. Mild chymotrypsin treatment of unsealed membranes releases the cytoplasmic domain into the soluble fraction, leaving two major integral fragments with molecular weights of 14 500–19 000 and 35 000–38 000 in the membranes (Figure 2; Steck et al., 1978; Reithmeier, 1979; Drickamer, 1978). With the exception of the N-terminal lysine residue, the sequence at the amino terminus of the 14 500–19 000-dalton fragment is identical with that of the 52 000–60 000-dalton tryptic fragment, and thus it appears that the Ch_2 chymotryptic site is a single residue before the T_1 tryptic site (Mawby & Findlay, 1982).

Chymotrypsin digestion of membranes that were prepared from ^{3}H-methylated intact cells results in the disappearance of band 3 protein and the appearance of a radioactive 35 000-dalton membrane-bound fragment representing the C-terminal chymotryptic fragment. This digestion also generated a 15 000-dalton membrane fragment which respresents the 14 500–19 000-dalton fragment expected. This fragment had no detectable radioactivity (data not shown). We were surprised by this latter result, because this fragment would be expected to contain all of the methylation sites (estimated at 20% of the total methyl groups) common to the tryptic 52 000–60 000-dalton C-terminal segment and the chymotryptic 55 000–59 000-dalton N-terminal segment. Unless the presence of the methyl ester can allow protease digestion at sites not normally cleaved under these conditions (for example, at Ch_3), these results suggest that the number of methylation sites in the central region of the polypeptide may have been

Table I: Inhibition of Carboxyl Methylation and D-Aspartic Acid β-[^{3}H]Methyl Ester Formation in Intact Erythrocytes Incubated with Adenosine and Homocysteine Thiolactone and Cycloleucine[a]

inhibitors added during incubation of cells	pmol of Me ester from band 3[b]/mg of total membrane protein	pmol of D-Asp β-[^{3}H]Me ester from band 3/mg of total membrane protein
(A) none (control)	2.6 (100%)[c]	0.23 (100%)[c]
(B) 5 mM each of adenosine and homocysteine thiolactone	0.14 (5.4)	<0.0046[d] (<2%)
(C) 50 mM cycloleucine	0.26 (10)	<0.0046[d] (<2%)

[a] Intact cells were incubated with L-[*methyl*-^{3}H]methionine in the absence (A) or the presence of inhibitors (B and C) as indicated. Membranes were prepared, and the incorporation of methyl esters was quantiated as described under Experimental Procedures. D-Aspartic acid β-[^{3}H]methyl ester formation was calculated as described in the legend of Figure 5. [b] In this particular experiment, the incorporation of methyl ester was found to be 18.0, 1.6, and 3.0 pmol of ester/mg of total membrane protein for the three samples, respectively. The percent of methyl ester associated with band 3 was determined from a fluorograph of the same lane to be 14.6%, 8.6%, and 8.8%, respectively. [c] The number in parentheses indicates the value relative to that of the control. [d] The limit of detection was estimated at 3 times the standard deviation of the background radioactivity.

overestimated.

An additional experiment was performed to generate the central fragment between T_1 and Ch_1. Membranes from chymotrypsinized ^{3}H-methylated intact cells were extracted with lithium diiodosalicylate to remove extrinsic proteins (Terwilliger & Clarke, 1981). The residual membrane pellets were digested with trypsin to produce an approximate 20 000-dalton fragment from the C-terminus of the 59 000-dalton peptide (Steck et al., 1978). Although we did detect a new radioactive band at 23 000 daltons in this experiment which may correspond to this central fragment, it is also possible that some or all of the radioactivity represents methylation sites from the C-terminus region since a minor site of trypsin cleavage about 20 000 daltons from the carboxyl terminal of the protein has been recently found (Jennings et al., 1986).

Isolation of D-*Aspartic Acid* β-[^{3}*H*]*Methyl Ester from Band 3 from Intact Erythrocytes Incubated with* L-[*methyl*-^{3}*H*]-*Methionine.* Acetic acid extracted ^{3}H-membranes were digested with carboxypeptidase Y, a protease that releases aspartic acid β-[^{3}H]methyl ester from erythrocyte membrane proteins (McFadden & Clarke, 1982; Clarke et al., 1984). The soluble products of the digestion were separated by cation-exchange chromatography on amino acid analysis resin as described under Experimental Procedures. A radioactive peak was found to coelute with an added standard of aspartic acid β-methyl ester. This radioactive material was further purified and desalted by gel filtration chromatography. The radioactive compound again coeluted with the aspartic acid β-methyl ester standard. This material was coupled to L-LeuCA in a reaction which produces diastereomeric L-leucyl dipeptides from a mixture of enantiomers (Manning & Moore, 1968). The resulting dipeptides were separated by ion-exchange chromatography using an amino acid analyzer resin (Figure 5, left). A new peak of radioactivity was found to coelute with the L-leucyl-D-aspartic acid β-methyl ester standard. There was also radioactivity coeluting with the unreacted starting material. No radioactivity was found to coelute with the L-leucyl-L-aspartic acid β-methyl ester standard. The recovery of the L-aspartic acid β-[^{3}H]methyl ester was calculated to be 0.23 pmol/mg of membrane protein; this corresponds to a yield of about 9% of the original band 3 radioactive methyl esters (Table I).

To show that the D-aspartic acid β-[^{3}H]methyl ester isolated from band 3 is a product of the erythrocyte protein carboxyl methyltransferase, parallel experiments were performed in which the methylation reaction was inhibited during the incubation of intact erythrocytes with L-[*methyl*-^{3}H]methionine. Methylation reactions were inhibited either under conditions where the product inhibitor *S*-adenosylhomocysteine was allowed to accumulate in the presence of adenosine and homocysteine thiolactone (Barber & Clarke, 1984) or when cycloleucine was used to inhibit the *S*-adenosylmethionine synthetase reaction. In both cases, the resulting acetic acid extracted pellets were analyzed for D-aspartic acid β-[^{3}H]-methyl ester content after proteolysis as above. No detectable radioactivity was found to coelute with an added standard of

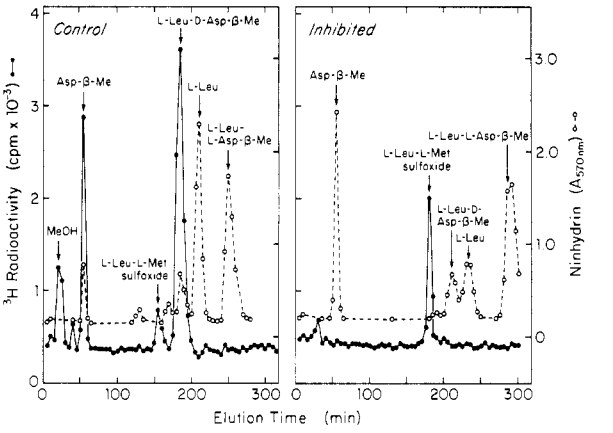

FIGURE 5: Isolation of D-aspartic acid β-[^{3}H]methyl ester from band 3 physiological methylation sites. (Left) Band 3 enriched ^{3}H-membranes were prepared and digested with carboxypeptidase Y as described under Experimental Procedures. The soluble fraction after digestion was subjected to ion-exchange chromatography using a column (0.9 × 30 cm) packed with amino acid analyzer resin (Durrum DC-6A, Dionex Chemical Corp.) eluted with pH 3.25 citrate buffer (0.2 M in Na^+). The peak containing aspartic acid β-methyl ester was pooled and desalted by Sephadex G-15 gel filtration in 0.1 M acetic acid. Again, the peak containing aspartic acid β-methyl ester was pooled. This lyophilized material was then coupled to L-LeuCA as described (Clarke et al., 1984). The products were separated by ion-exchange chromatography using a column (0.9 × 53 cm) packed with Beckman amino acid analyzer resin (AA-15) eluted with pH 3.25 citrate buffer (0.2 M in Na^+) at 56 °C. The elution profile from this column is shown on the left. The sum of the radioactivity coeluting with standards of aspartic acid β-methyl ester, L-leucyl-D-aspartic acid β-methyl ester, and methanol derived from hydrolysis was taken to represent the total amount of methyl ester applied to the column. Using the value of the specific activity of the L-[*methyl*-^{3}H]methionine used in the initial incubation, it was possible to calculate that the yield of D-aspartic acid β-methyl ester recovered from this band 3 preparation was 0.23 pmol/mg of membrane protein (see Table II). Radioactivity was detected by liquid scintillation counting of 2.4 mL of each fraction in 17 mL of ACS II (Amersham) (solid line), and the added amino acid standards were detected by ninhydrin assay of 0.7-mL aliquots of each fraction (dashed line). MeOH, methanol. (Right) Packed erythrocytes were incubated with 5 mM each of adenosine and homocysteine thiolactone as described under Experimental Procedures. After 1 h at 37° with shaking, samples were transferred to tubes containing lyophilized L-[*methyl*-^{3}H]methionine. Methylation was allowed to proceed or 4 h at 37 °C (13.2 μM methionine). Band 3 enriched vesicles were prepared and digested, and the digestion supernatants were analyzed exactly as described above.

Table II: D-Aspartic Acid β-Methyl Ester Isolation from Intracellular and Extracellular Methylation Sites

source of membranes	pmol of Me esters from band 3/mg of total membrane protein	pmol of D-Asp β-[^{3}H]Me ester from band 3/mg of total membrane protein	D-Asp β-[^{3}H]Me ester from band 3/total Me ester in band 3
trypsinized cells labeled with *S*-adenosyl[^{3}H]Met under lysis conditions (extracellular and intracellular sites)	40[a]	0.4	1/100 (1%)
intact erythrocytes incubated with L-[*methyl*-^{3}H]Met (intracellular sites)	2.6	0.23	1/11 (9%)

[a] Calculated from Terwilliger and Clarke (1981).

aspartic acid β-methyl ester in cation-exchange chromatography of digests from both adenosine and homocysteine thiolactone, and cycloleucine-treated samples. The results of the adenosine/homocysteine thiolactone experiment, in which the aspartic acid β-methyl ester fraction from the ion-exchange column was subjected to gel filtration chromatography and L-LeuCA analysis, are shown in Figure 5 (right). No radioactive product was found to coelute with either the diastereomeric dipeptides or the starting material. Similar results were obtained for the cycloleucine-treated samples. These data, summarized in Table I, indicate that the D-aspartic acid β-[^{3}H]methyl ester isolated from band 3 is a product of the erythrocyte protein carboxyl methyltransferase.

Isolation of D-*Aspartic Acid β-[^{3}H]Methyl Ester from Band 3 from Lysed Erythrocytes Incubated with S-Adenosyl-*L-*[methyl-^{3}H]methionine. Comparison of Extracellular and Intracellular Sites of Band 3.* When methylation reactions are performed under conditions where sites on the external surface of erythrocytes are available, a large increase in methyl ester incorporation is seen (O'Connor & Clarke, 1983). Therefore, when both the external and the internal sites are subjected to methylation (such as when lysed cells are incubated with *S*-adenosyl-[*methyl*-^{3}H]methionine; Terwilliger & Clarke, 1981), one might expect to detect an increase in the amount of D-aspartic acid β-[^{3}H]methyl ester in a carboxypeptidase Y digest of these membranes. The separation of ^{3}H-methylated band 3 from the other methylated species in membranes prepared from lysed erythrocytes incubated with *S*-adenosyl-[*methyl*-^{3}H]methionine (Terwilliger & Clarke, 1981) cannot be accomplished by simply removing the extrinsic polypeptides, because under these methylation conditions glycophorin A and other intrinsic membrane proteins are also methyl-accepting substrates for the protein carboxyl methyltransferase (Terwilliger & Clarke, 1981; O'Connor & Clarke, 1983). However, trypsin digestion of intact erythrocytes has been found to degrade all of the methyl acceptors of lysed membranes with the exception of band 3, and this procedure was used here to generate membranes containing methyl group radioactivity in band 3 alone (Figure 6). This band 3 preparation, which should contain both intracellular (physiological) and extracellular methylation sites, was then digested with carboxypeptidase Y as described above. Aspartic acid β-methyl ester was isolated and analyzed for its stereochemical configuration by the L-LeuCA derivatization method described above. Radioactive material that coeluted with an L-leucyl-D-aspartic acid β-methyl ester standard was detected, while no evidence was found for L-leucyl-L-aspartic acid β-[^{3}H]methyl ester (Table II).

The yield of D-aspartic acid β-[^{3}H]methyl ester from the trypsinized lysed cell preparation was compared to that from the intact cell preparation. Table II shows that, although band 3 can incorporate 8–9 times more methyl esters in a lysed cell incubation than in an intact cell incubation, this difference is not reflected in the amount of D-aspartic acid β-[^{3}H]methyl ester isolated. This result suggests that the extracellular methylation sites do not contribute to the formation of D-aspartic acid β-methyl esters.

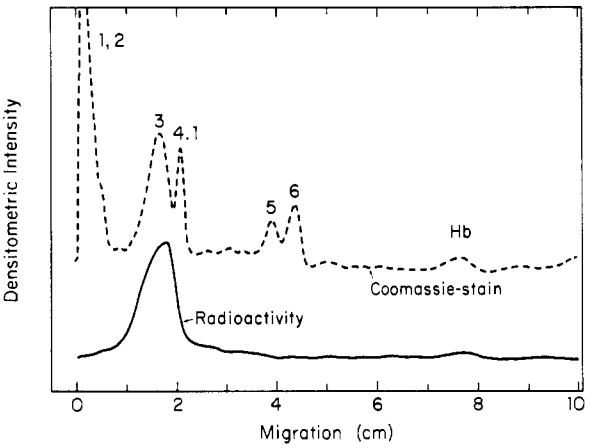

FIGURE 6: Membrane protein methylation in trypsinized lysed erythrocytes. Cells were trypsinized followed by lysing and incubation with *S*-adenosyl-L-[*methyl*-^{3}H]methionine as described under Experimental Procedures. Incorporation of [^{3}H]methyl ester into membrane proteins was analyzed by SDS/urea–polyacrylamide gel electrophoresis. A densitometric trace of the Coomassie-stained bands (dashed lines) was superimposed on a densitometric trace of a fluorogram of the same lane (solid line).

DISCUSSION

Location of Physiological Methylation Sites on the Band 3 Polypeptide. From the data presented here, there appear to be a minimum of three physiological methylation sites in band 3. At least one site is in the soluble cytoplasmic domain in the 22 000–23 000-dalton N-terminal fragment, while a second site (or sites) is located in the C-terminal 38 000-dalton chymotryptic fragment. On the basis of quantitation of methyl group incorporation, there also appear to be sites in the central region, although we have not confirmed these sites by direct mapping to a specific proteolytic fragment. No sites have been detected in sequences known to be embedded in the lipid bilayer.

The most important result of this work is that methylation sites appear to be located in many of the polypeptide regions that would be accessible to a cytosolic enzyme such as the protein carboxyl methyltransferase. The lack of specificity for a single site is consistent with the previous studies which have indicated that almost all proteins can be methylated to some extent by this class of enzymes (Clarke, 1985). Because L-isoaspartyl and D-aspartyl residues, the apparent substrate sites for this enzyme, could be derived in theory from any aspartyl or asparaginyl residue in a protein sequence, it is possible that there may be a large number of methylation sites on the band 3 polypeptide. The rate of formation of altered aspartyl residues may be very dependent upon both the sequence and three-dimensional structure surrounding the site and may vary for different residues in the linear sequence.

In previous work, it was suggested that band 3 methylated in a lysed cell preparation contained a methylation site(s) near the junction of the N-terminal cytoplasmic and membrane domain. Interestingly, no evidence was found in this previous study for the methylation of the N-terminal cytoplasmic domain (Terwilliger & Clarke, 1981).

Isolation of D-*Aspartic Acid β-[³H]Methyl Ester from Band 3.* In this work, we have shown that D-aspartic acid β-[³H]-methyl ester can be isolated from carboxypeptidase Y digests of band 3 from intact erythrocytes incubated with L-[*methyl*-³H]methionine and from trypsinized lysed erythrocytes incubated with *S*-adenosyl-L-[*methyl*-³H]methionine. The yield of this compound from intact cells was 9% of the total population of band 3 methyl esters but only 1% from the lysed cell preparation. The identity of the remaining population of methyl esters is unknown. The isolation and identification of carboxyl methylated residues from eucaryotes are complicated by the chemical lability of the methyl esters as well as by the lack of efficient cleavage by proteases of peptide bonds adjacent to altered aspartyl residues (Clarke et al., 1984). From studies using synthetic peptides and deamidated polypeptides, it is clear that L-isoaspartyl α-carboxyl groups can also be substrates for methylation (Murray & Clarke, 1984; Aswad, 1984; McFadden & Clarke, 1986). It is possible that such methylated sites are completely lost during proteolytic digestions because the α-methyl ester resembles the α-amide peptide bond and may be rapidly hydrolyzed by the protease. We have detected, however, a methylated product of protease digestion that appears to coelute with a standard of the aspartyl α-methyl ester of L-aspartyl-β-glycine. This product may represent a methylated L-isoaspartylglycyl sequence on band 3.

The origin of D-aspartic acid β-methyl ester residues is of considerable interest because synthetic peptides containing D-aspartyl residues have not yet been found to be substrates for preparations of the erythrocyte protein carboxyl methyltransferase (Murray & Clarke, 1984; J. Lowenson, T. Geiger, and S. Clarke, unpublished results). These results suggest that the methylation sites which give rise to D-aspartic acid β-methyl ester upon protease digestion may represent specialized sequences or conformations of intracellular proteins.

Physiological Implications. In a previous study, we have shown that inhibition of methylation reactions in intact erythrocytes for periods of up to 48 h has little or no effect on the physiological function of the band 3 protein in anion transport (Lou & Clarke, 1986). It appears unlikely, therefore, that the methylation of this protein has a regulatory role comparable to the modulation of chemoreceptor function in bacteria by the methylation of L-glutamyl residues (Stock et al., 1985a,b). This conclusion is also supported by the substoichiometric methylation of this protein, estimated at 1 methyl group per 500–600 molecules at steady state in intact erythrocytes (Freitag & Clarke, 1981; Barber & Clarke, 1983). From the results of this study, which show that there are at least 3 "physiological" methylation sites in the band 3 polypeptide, one can calculate that this level would be further reduced to 1 methyl group at a particular site per about 1500–1800 molecules. It is difficult to imagine how such a small degree of modification could affect the activity of a population of proteins. Second, none of the transmembrane fragments that are known to contain sites of anion and anion transport inhibitor binding (Drickamer, 1980; Rothstein et al., 1980) are methylated.

An alternative role for mammalian protein carboxyl methylation has been proposed recently (McFadden & Clarke, 1982; Clarke & O'Connor, 1983; Clarke, 1985). The apparent recognition of D-aspartyl and L-isoaspartyl residues by the methyltransferase may be the first step in a process by which such altered residues are converted back to normal configurations in a repair reaction or are recognized by proteolytic enzymes in a degradation reaction. The significance of these methylation reactions may become more important with time, because racemized or isomerized residues would otherwise tend to accumulate in cellular proteins. In this way, the function of a given protein would not necessarily be altered by the degree of covalent modification per se, but the lack of such modification would result in the absence of protein repair or degradation and may cause the eventual deterioration of protein activity due to an accumulation of the "damaged" protein population.

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Raman Microscope Studies on the Primary Photochemistry of Vertebrate Visual Pigments with Absorption Maxima from 430 to 502 nm†

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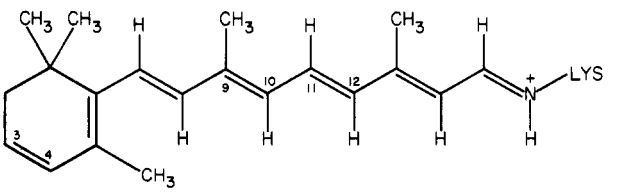
ABSTRACT: Raman microscope vibrational spectra have been recorded from single photoreceptor cells frozen at 77 K. Spectra of photostationary steady-state mixtures of visual pigments and their primary photoproducts were obtained from toad red rods (λ_{max} 502 nm), angelfish rods (λ_{max} 500 nm), gecko blue rods (λ_{max} 467 nm), and bullfrog green rods (λ_{max} 430 nm). All four photoproducts have enhanced low-wavenumber Raman lines at ~850, 875, and 915 cm^{-1} and show the anomalous decoupling of the 11- and 12-hydrogen out-of-plane (HOOP) wagging vibrations, as is observed in the bovine primary photoproduct. The low-wavenumber lines are enhanced in the resonance Raman spectrum by conformational distortion, and the uncoupling of the 11- and 12-hydrogen wags is caused by additional protein perturbations. The similarity of the HOOP modes in all four photoproducts indicates that the protein perturbations that uncouple the 11- and 12-hydrogen wags and that enhance the HOOP modes are very similar. Thus, these perturbations of the photoproduct Raman spectrum cannot be caused by the same protein–chromophore interactions that are responsible for wavelength regulation in these pigments.

Vision begins with the absorption of light by the visual pigments of the retina [for a review see Birge (1981)]. The chromophore of visual pigments is either 11-*cis*-retinal (A_1 retinal) or its 3,4-didehydro derivative (A_2 retinal) that is covalently bound as a protonated Schiff base to a lysine residue of the protein opsin (Oseroff & Callender, 1974):



Although the 11-cis A_1-protonated Schiff base absorbs at 440 nm in ethanol, A_1 visual pigments have absorption maxima that vary from about 430 to 562 nm (Dartnall & Lythgoe, 1965). This variation in pigment absorption spectrum is vital for the perception of color and for visual discrimination under a wide range of ambient light conditions (Jacobs, 1976; Levine & MacNichol, 1979). Modification of pigment absorption spectra must arise from alterations in protein sequence and differences in protein–chromophore interactions (Nathans et al., 1986).

Several types of mechanisms have been proposed to explain the molecular basis of these spectral changes, known as "opsin shifts". The protein could regulate the spectral sensitivity of the pigment by introducing ground-state twists (Blatz & Liebman, 1973), by affecting the polarizability or dielectric constant of the chromophore's environment (Blatz & Mohler, 1975; Irving et al., 1970), or by electrostatically perturbing the chromophore with charged amino acid residues (Kropf & Hubbard, 1958; Honig et al., 1976). Also, alterations in pigment spectral sensitivity could be brought about through regulation of the distance between the Schiff base nitrogen and its counterion (Blatz & Mohler, 1975; Blatz et al., 1972).

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