Biochimica et Biophysica Acta 903 (1987) 37–47
Elsevier

BBA73660

Carboxypeptidase Y digestion of band 3, the anion transport protein of human erythrocyte membranes

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(Received 10 December 1986)
(Revised manuscript received 10 February 1987)

Key words: Carboxypeptidase Y; Band 3 protein; Anion transport; (Human erythrocyte membrane)

The exposure of the carboxyl-terminal of the Band 3 protein of human erythrocyte membranes in intact cells and membrane preparations to proteolytic digestion was determined. Carboxypeptidase Y digestion of purified Band 3 in the presence of non-ionic detergent released amino acids from the carboxyl-terminal of Band 3. The release of amino acids was very pH dependent, digestion being most extensive at pH 3, with limited digestion at pH 6 or above. The 55000 dalton carboxyl-terminal fragment of Band 3, generated by mild trypsin digestion of ghost membranes, had the same carboxyl-terminal sequence as intact Band 3, based on carboxypeptidase Y digestion. Treatment of intact cells with trypsin or carboxypeptidase Y did not release any amino acids from the carboxyl-terminal of Band 3. In contrast, carboxypeptidase Y readily digested the carboxyl-terminal of Band 3 in ghosts that were stripped of extrinsic membrane proteins by alkali or high salt. This was shown by a decrease in the molecular weight of a carboxyl-terminal fragment of Band 3 after carboxypeptidase Y digestion of stripped ghost membranes. No such decrease was observed after carboxypeptidase Y treatment of intact cells. In addition, Band 3 purified from carboxypeptidase Y-treated stripped ghost membranes had a different carboxyl-terminal sequence from intact Band 3. Cleavage of the carboxyl-terminal of Band 3 was also observed when non-stripped ghosts or inside-out vesicles were treated with carboxypeptidase Y. However, the digestion was less extensive. These results suggest that the carboxyl-terminal of Band 3 may be protected from digestion by its association with extrinsic membrane proteins. We conclude, therefore, that the carboxyl-terminal of Band 3 is located on the cytoplasmic side of the red cell membrane. Since the amino-terminal of Band 3 is also located on the cytoplasmic side of the erythrocyte membrane, the Band 3 polypeptide crosses the membrane an even number of times. A model for the folding of Band 3 in the erythrocyte membrane is presented.

Introduction

The Band 3 protein of the erythrocyte membrane catalyzes the one-for-one exchange of anions, a process necessary for respiration [1,2]. This glycoprotein ($M_r = 95000$) is the major intrinsic protein of the erythrocyte membrane. The complete amino acid sequence of mouse Band 3 has recently been deduced from the nucleotide sequence of cDNA transcribed from mRNA for Band 3 [3,4]. Band 3 is composed of two domains [3–13]. The amino terminal domain ($M_r = 41000$) is exposed to the cytoplasm of the cell and can be released from the membrane by mild proteolytic cleavage as a water-soluble fragment [5]. The

Abbreviation: $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether.

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amino-terminal of Band 3 is clearly located on the cytoplasmic side of the erythrocyte membrane [1,2]. The cytoplasmic domain provides the binding sites for the cytoskeleton [14], hemoglobin [15,16] and enzymes such as aldolase and glyceraldehyde-3-phosphate dehydrogenase [17–20]. The carboxyl-terminal domain (M_r = 55 000) is located in the membrane and is capable of carrying out anion transport [21]. Analysis of the amino acid sequence, proteolytic digestion and chemical labelling studies have shown that this portion of Band 3 spans the membrane up to 12 times [3–5,11–13,22–42]. These transmembrane sequences are arranged in an α-helical conformation [10]. The location of the carboxyl-terminal of Band 3 with respect to the membrane is not known [1,3,4,26,30,31,35,36,40,41]. Many models indicate that the carboxyl-terminal of Band 3 is on the outside of the red cell [5,32,37–39]. This was based on the assumption that this part of Band 3 is sensitive to external chymotrypsin [5], an assertion that was later proved to be incorrect [9,21]. In this paper, we provide evidence that the carboxyl-terminal of Band 3 is located on the cytoplasmic side of the erythrocyte membrane.

Experimental Procedures

Materials. Carboxypeptidase Y (58–104 units per mg) was obtained from Worthington Diagnostic Systems Inc. and was dissolved in distilled water at 1 mg/ml. TPCK-treated trypsin and TLCK-treated chymotrypsin were obtained from Sigma Chemical Company. Octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) was purchased from Nikko Chemical Co., Tokyo. Outdated human red blood cells were kindly provided by the Canadian Red Cross. All chemicals were of pure reagent grade.

Methods. All steps were performed at 0–4 °C, unless stated otherwise. Erythrocytes were washed three times with 0.9 percent NaCl. Cells were collected by centrifugation at 5000 rpm for 10 min and the buffy coat was removed by aspiration. Ghost membranes were prepared by hypotonic lysis in 5 mM sodium phosphate (pH 8.0) [43]. Stripped membranes were prepared by extracting membranes with 10 volumes of 2 mM EDTA, pH 12 at 0 °C [5]. Membranes were collected by centrifugation at 15 000 rpm in an SS-34 rotor in a Sorvall RC-5B centrifuge.

Band 3 was purified as described previously [44,45]. Briefly, ghost membranes depleted of Band 6 or EDTA-stripped membranes were solubilized with 1–5 volumes of 1 percent $C_{12}E_8$ in 5 mM sodium phosphate (pH 8.0) at 0 °C for 20 min. Band 3 was purified by ion-exchange on aminoethyl-Sepharose 4B followed by chromatography on ((*p*-chloromercuri)benzamido)ethylene) amino-Sepharose 4B (PCMB-Sepharose) [44,45]. The 55 000 dalton membrane associated domain was purified as described previously [10]. Briefly, this fragment was generated by treating membranes with 5 μg/ml of trypsin at 0 °C for 1 h. Extrinsic fragments were extracted with 2 mM EDTA (pH 12). The 55 000 dalton fragment was solubilized with $C_{12}E_8$ and purified by ion-exchange chromatography on DEAE CL-Sepharose [10].

Carboxypeptidase Y [46,47] digestions of purified Band 3 and the 55 000 dalton fragments were performed in 0.1 percent $C_{12}E_8$, 5 mM sodium phosphate (pH 3.0) at room temperature for various times up to 2 h. Norleucine was added as an internal standard (1 nmol/nmol Band 3). Blank samples containing Band 3 but not carboxypeptidase and carboxypeptidase without Band 3 were also analyzed. The effect of pH on the release of amino acids from Band 3 was performed in the same buffer at pH values of 2 to 6. The digestion was stopped by addition of an equal volume of 10 percent trichloroacetic acid and cooling to 0 °C. The precipitated protein was removed by centrifugation and the trichloroacetic acid was removed by extraction with ether. Samples were freeze-dried and subjected to amino acid analysis using a Durrum Amino Acid Analyzer. Asparagine eluted at the serine position and was quantitated by the decrease in the serine peak and the increase in the aspartate peak after hydrolysis of the sample in 6 M HCl at 115 °C for 16 h.

The following procedures were followed for the treatment of intact cells and membranes with proteases and the subsequent purification of Band 3 for carboxyl-terminal analysis. Intact erythrocytes were treated with carboxypeptidase Y at 0–10 units/ml for 1–18 h at pH values of 4.5–6.0 at room temperature in 205.3 mM sucrose, 28.5 mM

sodium citrate. Trypsin digestion of cells at a 50 percent hematocrit was performed in 150 mM NaCl, 5 mM sodium phosphate (pH 8.0) at 37 °C for 1 h using 1 mg/ml trypsin. Trypsin digestion was stopped by addition of PMSF to 1 mM and further incubation at 37 °C for 15 min. The cells were then washed with 150 mM NaCl, 5 mM sodium phosphate, (pH 8.0) buffer containing 0.5 percent fatty acid free bovine serum albumin, followed by two washes of buffer only. Trypsin digestion of ghost membranes was carried out in 5 mM sodium phosphate (pH 8.0) at 0 °C for 1 h with 5 μg/ml of enzyme. Carboxypeptidase Y treatments of ghost and stripped membranes were performed in 5 mM sodium phosphate at room temperature at an enzyme to protein ratio of 1/200 (1 units/ml) at pH values of 3.5 to 6.0. Inside-out vesicles were prepared according to Steck and Kant [48].

The following procedure was employed for the small scale treatment of intact cells and ghost membranes with carboxypeptidase Y for subsequent analysis by sodium dodecyl sulfate gel electrophoresis. Erythrocytes at a 50 percent hematocrit in 205.3 mM sucrose, 28.5 mM sodium citrate (pH 4.5–6.0) were treated with 5–10 units/ml carboxypeptidase Y at 37 °C for 1 h. Cells were then washed once with the same buffer then with 150 mM NaCl, 5 mM sodium phosphate (pH 7.4) twice. Ghosts or ghosts stripped of extrinsic membrane proteins with 2 mM EDTA (pH 12) or with 0.2 mM EDTA, 7.5 mM sodium phosphate (pH 7.5) at 37 °C for 20 min followed by 1 M KI, 1 mM EDTA, 7.5 mM sodium phosphate (pH 7.5) at 37 °C for 20 min were treated with carboxypeptidase Y (5 units/ml) at 37 °C for 1 h at pH values of 3.5, 4.5, 5.5 and 6.5.

In order to visualize the carboxyl-terminal 35000 dalton fragment of Band 3 on sodium dodecyl sulfate gels the erythrocytes were treated with chymotrypsin and endo-β-galactosidase as described by Jennings et al. [26]. Briefly, cells were treated with 0.5 mg/ml chymotrypsin at 37 °C for 2 h in 150 mM NaCl, 5 mM sodium phosphate (pH 7.4) at a 50 percent hematocrit. After extensive washing [26] to remove chymotrypsin, the cells were treated with 10 mU/ml of endo-β-galactosidase in 150 mM KCl, 10 mM sodium citrate (pH 6.0) at 37 °C for 1 h. Alternatively, ghosts prepared from chymotrypsinized cells were treated with endo-β-galactosidase treatment at 10 mU/ml in 10 mM sodium citrate (pH 5.7) at 37 °C for 1 h. Ghost membranes were isolated as described above, stripped with 2 mM EDTA (pH 12) and then solubilized with Laemmli sample buffer [49].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Laemmli [49]. Protein concentrations were determined according to Lowry et al. [50], except that all samples were solubilized with 1 percent sodium dodecyl sulfate.

Results and Discussion

Valine is the carboxyl-terminal amino acid in human and mouse Band 3 [3,11,26]. Drickamer reported that valine was released from human Band 3 by hydrazinolysis and that a small amount of valine was also released from human Band 3 using carboxypeptidase A [11]. We were unable to detect the release of any valine from purified Band 3, using carboxypeptidase A at an enzyme to substrate ratio of 1/100 at 37 °C in 0.1 percent $C_{12}E_8$, 0.1 M *N*-ethylmorpholine acetate (pH 8.0). In addition, carboxypeptidase B failed to release any amino acids from Band 3 under similar digestion conditions. Jennings et al. [26] found that carboxypeptidase Y released 0.76 moles of valine per mole of Band 3 after a 3–5 h digestion period in 0.1 percent sodium dodecyl sulfate, 50 mM sodium citrate (pH 6.1) at 22 °C using a very high enzyme to protein ratio (1/16–1/42) [1].

We have investigated the digestion of purified Band 3 by carboxypeptidase Y in the presence of $C_{12}E_8$, a mild non-ionic detergent. This detergent does not alter significantly the native structure of the Band 3 protein [10,44]. The time-course of release of amino acids from Band 3 by carboxypeptidase Y is shown in Fig. 1. The values are the average of the digestion of four Band 3 preparations.

At pH 3.0, carboxypeptidase Y at a 1:200 enzyme to protein ratio readily released amino acids from the carboxyl terminal of purified Band 3 in the presence of 0.1 percent $C_{12}E_8$ (Fig. 1). Valine was the most rapidly released amino acid, consistent with valine being the carboxyl-terminal

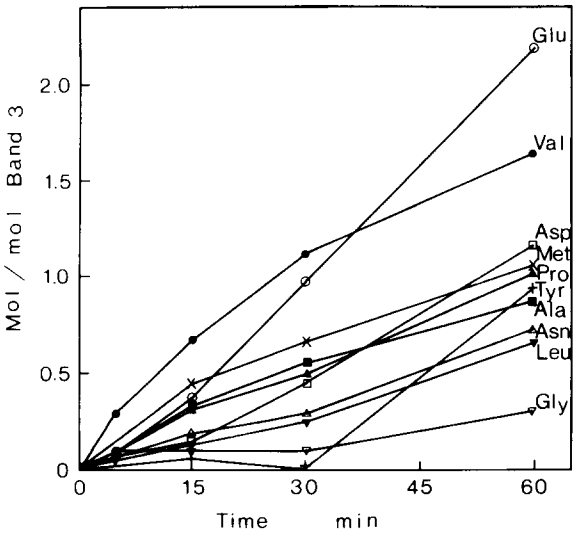

Fig. 1. Kinetics of carboxypeptidase Y digestion of purified Band 3 in 0.1 percent $C_{12}E_8$, 5 mM sodium phosphate (pH 3.0) at 22°C. The yields of amino acids are the average of carboxypeptidase Y digestion of four preparations of purified Band 3. The protein concentrations were 2.5, 2.6, 3.2 and 1.7 mg/ml. Carboxypeptidase Y was added at a ratio of 1:200 to Band 3 protein.

residue in Band 3 [3,11,36]. The kinetics of release of amino acids from Band 3 by carboxypeptidase Y did not allow us to establish the amino acid sequence at the carboxyl terminus of Band 3 with certainty. The digestion pattern was, however, consistent with the sequence of amino acids at the carboxyl terminal of mouse Band 3, with one or two exceptions. The carboxyl-terminal sequence of mouse Band 3 is [3]

-Glu-Asn-Gly-Leu-Asp-Glu-Tyr-Asp-Glu-Val-Pro-Met-Pro-Val

There was some variability in the yields of amino acids released from Band 3 by carboxypeptidase Y digestion. For example, the yields of valine after a 60 min digestion at 22°C and pH 3.0 were 1.91, 2.09, 0.90 and 1.60 moles of valine per mole of Band 3. This variability may be due to differences in the activity of the carboxypeptidase Y, the purity of the Band 3 preparation, endolytic cleavage and release from internal sequences and the extent of digestion. The results are, however, in good agreement with the expected stoichiometry of released amino acids based on the mouse sequence. Carboxypeptidase Y at pH 3 released an average of 1.63 moles of valine per mole of Band 3, while glutamic acid was released in slightly over two moles per mole of Band 3 over a 1 h digestion period at 22°C (Fig. 1). Aspartate, methionine, proline, alanine and tyrosine were released in close to 1 mol/mol of Band 3. Lower amounts of leucine, asparagine (at serine position of amino acid analyzer) and glycine were released in the 1 h period. Threonine, isoleucine, phenylalanine, histidine, lysine and arginine were released in less than 0.1 mol/mol of Band 3.

One clear difference exists between the human and mouse carboxyl-terminal sequence. The mouse sequence contains two proline residues, two and four residues from the carboxyl terminus [3]. Only 1 mole of proline is released from human Band 3 by carboxypeptidase Y. An alanine is released from human Band 3 in stoichiometric amounts although no alanine is present in the carboxyl-terminal region of the mouse sequence. We propose that one proline in the mouse sequence is replaced by an alanine residue in the human sequence. This could be accounted for by a single base change in the codons for proline [3] from CCC or CCG to GCC or GCG, respectively, for alanine. A second possible difference in the mouse and human carboxyl-terminal sequences is the position of the leucine residue. We find that this leucine is released before a second mole of aspartate is released. It is possible that this leucine residue is more carboxyl terminal in the human sequence or that the aspartate following the leucine residue in the mouse sequence is replaced by a glutamate in the human sequence.

The rate and extent of the digestion of Band 3 by carboxypeptidase Y was very pH dependent, release being very slow at pH values of 6 and above (Table I). The results are shown using the sequence of mouse Band 3 with the exception of an alanine residue four residues from the carboxyl terminus rather than a proline residue. A single preparation of Band 3 was used in this experiment in contrast to the data given in Fig. 1 which are the average yields of amino acids released by carboxypeptidase Y digestion of four different preparations of Band 3. There is therefore not perfect agreement between the data presented in Fig. 1. and Table I. Digestion was most rapid at pH 3.0. No release of amino acids was observed at

TABLE I

pH DEPENDENCE OF CARBOXYPEPTIDASE Y DIGESTION OF PURIFIED BAND 3

Carboxypeptidase Y (1.3 U/ml) digestion of Band 3 (2.5 mg/ml) was performed at 22° C at an enzyme to protein ratio of 1/200 in 5 mM sodium phosphate at the indicated pH values.

Mouse Band 3 sequence (human)		Phe-	Asp-	Glu-	Glu-	Asn-	Gly	Leu-	Asp-	Glu-	Tyr-	Asp-	Glu-	Val-	Pro- (Ala) [a]	Met-	Pro-	Val-COO
pH	Time (min)	Mol amino acid/mol Band 3																
2.0	5–60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3.0	5	0	0	0	0	0.16	0.16	0.07	0	0	0	0.18	0.07	0	0.15	–	–	0.32
	15	0	0	0	0	0.31	0.14	0.14	0	0	0.09	0.26	0.40	0	0.47	0.49	0.38	0.98
	30	0	0	0	0	0.37	0.08	0.29	0	0	0	0.62	1.00	0.61	0.74	0.73	0.57	1.00
	60	0	0	0	0.54	0.71	1.00	0.70	0.39	1.00	0.94	1.00	1.00	0.91	1.27	1.47	1.67	1.00
4.0	60	0	0	0	0	0.34	0.24	0.51	0.10	0.35	1.08	1.00	1.00	0.91	1.14	0.88	1.49	1.00
5.0	120	0	0	0	0	0	0	0	0	0	0.44	0.47	0.65	0.76	1.04	0.84	1.60	1.00
6.0	60	0	0	0	0	0	0	0	0	0	0	0.06	0.04	0	0.12	0.22	0.27	0.36

[a] One of the proline residues in the mouse sequence is an alanine residue in human Band 3.

pH 2.0, presumably due to denaturation of the carboxypeptidase. At pH 3.0, carboxypeptidase Y (enzyme to protein ratio of 1 : 200 at room temperature for 1 h) cleaved the terminal 14 residues from Band 3 (Table I). At pH 4.0 digestion proceeded to residue 9 from the carboxyl terminus during the 1 h digestion period. At pH 5.0 digestion proceeded to residue 7 during a 2 h digestion period. Digestion at pH 6.0 was quite inefficient although partial removal of the terminal 3 residues occurred over a 1 h period (Table I). These results are entirely consistent with the specificity of carboxypeptidase Y [34,35]. Release of acidic amino acids occurs more rapidly under acidic conditions, therefore, digestion through the clustered acidic residues in the Band 3 sequence [3] would be rate limiting at more alkaline pH. In addition, release of amino acids from penultimate glycine residues is very slow. Therefore, cleavage of the glycine-leucine bond would also be expected to be slow.

The carboxyl-terminal sequence of mouse Band 3 is very hydrophilic and contains a number of lysine and arginine residues that should be sensitive to trypsin cleavage [3]. Trypsin treatment of red cells does not produce any proteolytic cleavage of Band 3 that is detectable by sodium dodecyl sulfate gel electrophoresis [5,11]. This does not preclude that trypsin removes a small peptide from the carboxyl terminus, since such a cleavage might not alter the mobility of Band 3 on gel electrophoresis significantly. Drickamer [11] has reported that trypsin applied to the exterior of erythrocytes does not digest the carboxyl terminal of Band 3. To confirm this observation, Band 3 was isolated from trypsin treated cells and subjected to carboxypeptidase Y digestion. The release of amino acids from this preparation of Band 3 (Table II) was similar to the release from control preparations of Band 3 (Fig. 1). For example, the yield of valine (1.60 mol/mol Band 3)) was indistinguishable from the average yield of valine from intact Band 3(1.63 mol/mol, Fig. 1). If trypsin had cleaved Band 3, the newly formed terminal lysine or arginine residue would not be sensitive to carboxypeptidase Y digestion at acid pH [46,47]. No amino acids should therefore have been released from Band 3 by the subsequent carboxypeptidase Y digestion. This experiment shows that trypsin applied to intact cells does not

TABLE II

CARBOXYPEPTIDASE Y DIGESTION OF BAND 3 PURIFIED FROM PROTEASE-TREATED CELLS

Carboxypeptidase Y (0.75 U/ml) digestion of purified Band 3 (1.5 mg/ml) was performed at 22° C at an enzyme to protein ratio of 1/200 in 5 mM sodium phosphate (pH 3.0). n.d., not determined.

Amino acid	mol/mol Band 3 Trypsin-treated cells [a] time (min)		Carboxypeptidase Y treated cells [b] time (min)		
	15	60	5	15	60
Asp	n.d.	0.64	0.12	0.49	0.98
Thr	0.38	n.d.	0.0	0.0	0.0
Ser	0.10	0.42	n.d.	0.12	0.15
Glu	0.42	0.80	n.d.	0.40	0.93
Pro	0.58	0.76	0.22	0.56	0.83
Gly	0.0	0.08	0.15	0.12	0.15
Ala	0.53	0.83	0.24	0.70	1.00
Val	1.06	1.60	0.54	1.50	1.89
Met	n.d.	0.48	0.20	0.52	0.68
Ile	0.0	0.09	0.0	0.0	0.0
Leu	0.42	0.89	0.0	0.0	0.09
Tyr	0.0	0.42	0.0	0.15	0.46
Phe	0.0	0.0	0.0	0.0	0.0
His	0.0	0.0	n.d.	n.d.	n.d.
Lys	0.0	0.0	n.d.	n.d.	n.d.
Arg	0.0	0.0	n.d.	n.d.	n.d.

[a] cells at a 50% hematocrit were treated with 1 mg/ml trypsin in 150 mM NaCl, 5 mM sodium phosphate (pH 8.0) at 37° C for 1 h.

[b] cells at a 25% hematocrit in 150 mM NaCl, 5 mM sodium phosphate (pH 6.0) were treated with 10 μg/ml (1 U/ml) carboxypeptidase Y at 37° C for 1 h.

cleave Band 3 and it eliminates the possibility that external trypsin removes a small peptide from the carboxyl-terminus of Band 3. This confirms that the carboxyl-terminal of Band 3 is not accessible to vigorous trypsin digestion in intact cells.

Mild trypsin treatment of ghost membranes (5 μg trypsin/ml, 0° C, 1 h) quantitatively cleaves the amino-terminal cytoplasmic domain (M_r = 41 000) from Band 3, leaving a 55 000 dalton carboxyl-terminal fragment in the membrane [5]. It is possible that under these conditions trypsin may also remove a small peptide from the carboxyl terminal of Band 3 that is not detectable by sodium dodecyl sulfate gel electrophoresis. The 55 000 dalton fragment was generated as described above and was purified. Carboxypeptidase Y digestion of this fragment revealed that most of the 55 000 dalton fragment contained an intact carboxyl terminal (Table III). Partial tryptic digestion of the carboxyl region of Band 3 may have occurred under these conditions since the yield of valine (1.35 mol/mol versus 1.63 mol/mol) and other amino acids was lower than usually found in control preparations of Band 3 (Fig. 1 and Table I). Small amounts of lysine and arginine were also released by carboxypeptidase Y (Table III). It is possible that the carboxyl-terminal region of Band 3 could be cleaved more efficiently at higher trypsin concentrations. Such conditions, however, would not result in the production of a large well-defined proteolytic fragment that could be isolated and redigested. The fact that mild trypsin treatment at 0° C of ghost membranes may partially cleave the carboxyl terminal of Band 3 while a 200-fold higher concentration of trypsin at 37° C does not cleave Band 3 when applied to intact cells suggests that the carboxyl terminal of Band 3 may be sensitive to trypsin in ghosts but not in cells.

TABLE III

CARBOXYPEPTIDASE Y DIGESTION OF THE 55000 DALTON CARBOXYL-TERMINAL TRYPTIC FRAGMENT

Carboxypeptidase Y (0.75 U/ml) digestion of the 55000 dalton tryptic fragment (1.5 mg/ml) was performed at 22° C at an enzyme to protein ratio of 1/200 in 5 mM sodium phosphate (pH 3). n.d., not determined.

Amino acid	mol/mol 55000 dalton fragment, time (min)	
	15	60
Asp	0.41	0.64
Thr	n.d.	0.20
Ser	n.d.	0.39
Glu	0.54	1.01
Pro	0.45	0.63
Gly	0.08	0.25
Ala	0.48	0.71
Val	0.84	1.35
Met	0.44	0.52
Ile	0.0	0.13
Leu	0.15	0.52
Tyr	0.0	n.d.
Phe	0.0	n.d.
His	0.0	0.0
Lys	0.0	0.16
Arg	0.08	0.19

In the next series of experiments intact cells and various membrane preparations were treated with carboxypeptidase Y. Band 3 was then isolated from these preparations and its carboxyl-terminal sequence was determined by a second carboxypeptidase Y digestion. If the initial treatment with carboxypeptidase Y had removed amino acids from the carboxyl terminal of Band 3, the release of amino acids by the second digestion would be different from digestion of control preparations of Band 3. In particular, the amount of valine released by the second carboxypeptidase treatment was an indication of the accessibility of the carboxyl terminal of Band 3 in the membrane preparations.

The sensitivity of the carboxyl terminal of Band 3 to carboxypeptidase Y in intact cells was examined. Band 3 isolated from carboxypeptidase-Y-treated erythrocytes was redigested with this enzyme. 1.89 moles of valine were released at 60 min from this preparation of Band 3 (Table II). The yield of leucine (0.09 moles/mole Band 3) was, however, much lower than usually found. The yield of leucine from four control preparations of Band 3 were 1.08, 0.70, 0.54 and 0.39 moles per mole of Band 3 (ave. = 0.68 mol/mol, Fig. 1). We conclude that carboxypeptidase Y did not digest as far into the Band 3 sequence in this case, stopping after the tyrosine residue. This may be due to the slow cleavage of the pair of acidic residues that preceed the leucine residue. The release of other amino acids up to the tyrosine residue was, however, entirely consistent with externally-applied carboxypeptidase Y having no effect on the carboxyl terminal of Band 3. The conditions used to digest intact cells (pH 6.0) would result in the partial removal of three or four amino acids from the carboxyl terminal of Band 3 (Table I). In addition, carboxypeptidase Y treatment of cells was performed at 37 °C rather than 22 °C to promote more vigorous digestion. Carboxypeptidase Y treatments of intact cells were also performed at lower pH values where more extensive digestion might occur (see below).

Treatment of ghost membranes with carboxypeptidase Y at pH 8.0 produced no detectable digestion of the carboxyl terminal of Band 3 (Table IV). The levels of valine (1.52), proline (0.86), methionine (0.86) and alanine (0.72) were similar to control preparations (Fig. 1). The same treatment at pH 5.0 resulted in more efficient digestion of Band 3 (Table IV). 1.34 moles of valine were released by the second carboxypeptidase Y treatment and the levels of proline (0.42 mol/mol), and alanine (0.62 mol/mol) were lower than found in control preparations. A similar result was obtained when stripped membranes were treated with carboxypeptidase Y at pH 6.0 (Table IV). In this case the yield of valine was only 1.24 mol/mol of Band 3 (Table IV). Carboxypeptidase Y digestion of ghost preparations at pH 5.0 or of stripped ghosts at pH 6.0 produced limited digestion of Band 3. This is a consequence of the pH dependence of carboxypeptidase digestion of Band 3 (Table I). In addition access of the enzyme to Band 3 might be restricted in the membrane as compared to Band 3 in solution. Ghosts were therefore stripped of extrinsic proteins and treated with carboxypeptidase at low pH.

Digestion of stripped ghosts with carboxypeptidase Y at pH 3.7 profoundly affected the carboxyl terminal of Band 3. Small amounts of aspartate, leucine and asparagine and stoichiometric amounts of glycine were released by the second carboxypeptidase Y digestion of Band 3. These results are consistent with carboxypeptidase Y removing the terminal ten residues from Band 3 in stripped ghosts at pH 3.7 (see sequence). This is completely consistent with the activity of the enzyme on purified Band 3 at low pH (Table I).

Inside-out vesicles treated with carboxypeptidase Y at pH 6.0 resulted in some cleavage of Band 3. 1.38 moles of valine per mole of Band 3 were released by the subsequent carboxypeptidase Y digestion (Table IV). The levels of proline, methionine and alanine were also slightly lower than normal (Table IV).

These results show that the carboxyl terminus of Band 3 is readily digested by carboxypeptidase Y at acidic pH in ghost membranes stripped of extrinsic membrane proteins. Limited digestion occurred at higher pH values in all ghost membrane preparations, consistent with the sensitivity of Band 3 to carboxypeptidase Y (Table I). In contrast, no digestion was observed by carboxypeptidase Y digestion of intact cells.

It is apparent from the experiments described above that carboxypeptidase Y is capable of re-

TABLE IV

CARBOXYPEPTIDASE Y DIGESTION OF BAND 3 PURIFIED FROM CARBOXYPEPTIDASE Y-TREATED MEMBRANE PREPARATIONS

Carboxypeptidase Y digestions of Band 3 (0.6–2.1 mg/ml) were performed at 22° C at an enzyme to protein ratio of 1/200 in 5 mM sodium phosphate (pH 3.0). n.d., not determined.

Amino acid	mol/mol Band 3											
	Carboxypeptidase Y-treated ghosts [a]						Carboxypeptidase Y-treated stripped ghosts b			Carboxypeptidase Y-treated inside-out vesicles [c]		
	pH 8.0, time (min)			pH 5.0, time (min)			pH 6.0, time (min)		pH 3.7, time (min)	pH 6.0, time (min)		
	5	15	60	5	15	60	15	60	60	5	15	60
Asp	0.0	n.d.	1.01	0.10	0.31	0.93	0.26	0.49	0.22	n.d.	0.41	0.84
Thr	0.0	0.0	0.0	0.0	0.0	0.0	0.09	0.14	n.d.	0.0	0.0	0.0
Ser	0.19	0.27	1.26	0.17	0.25	0.24	0.19	0.34	0.30	0.12	0.31	0.52
Glu	0.12	0.50	1.79	0.14	0.41	1.43	0.29	0.64	0.0	0.11	0.57	1.54
Pro	0.22	0.50	0.86	n.d.	0.19	0.42	0.41	0.78	0.0	0.23	0.52	0.68
Gly	0.17	0.13	0.09	0.12	0.17	0.17	0.18	0.20	0.81	0.14	0.19	0.12
Ala	0.21	0.47	0.72	0.14	0.33	0.62	0.37	0.74	0.03	0.17	0.48	0.67
Val	0.47	1.00	1.52	0.27	0.47	1.34	0.68	1.24	0.10	0.45	1.00	1.38
Met	0.32	0.81	0.89	0.13	0.26	0.66	0.30	0.55	n.d.	0.30	0.45	0.68
Ile	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.09
Leu	0.14	0.22	0.56	0.09	0.20	0.56	0.30	0.64	0.27	0.12	0.24	0.62
Tyr	0.0	0.14	0.37	0.0	0.0	0.26	0.08	0.22	0.0	0.0	0.08	0.34
Phe	0.0	0.09	0.01	0.0	0.0	0.0	0.04	0.10	0.0	0.0	0.0	0.0
His	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0	n.d.	n.d.	n.d.
Lys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0	n.d.	n.d.	n.d.
Arg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0	n.d.	n.d.	n.d.

[a] Ghosts at a protein concentration of 2.3 mg/ml were treated with carboxypeptidase Y (2.5 U/ml) at an enzyme to protein ratio of 1/100 in 5 mM sodium phosphate (pH 5.0 or 8.0) at 22° C for 2 h.

[b] Ghosts stripped of extrinsic proteins with 2 mM EDTA (pH 12) were treated with carboxypeptidase Y at an enzyme to protein ratio of 1/200 at pH 6.0 (98 mg protein) at 37° C for 1 h or pH 3.7 (136 mg protein) at 22° C for 1 h.

[c] Inside-out vesicles [48] at a protein concentration of 4.6 mg/ml were treated with carboxypeptidase Y (5 U/ml) at an enzyme to protein ratio of 1/200 in 0.5 mM sodium phosphate (pH 6.0) at 22° C for 1 h.

moving greater than ten residues from the carboxyl terminal of Band 3 under the appropriate acidic digestion conditions. The loss of this number of residues from the carboxyl terminal of Band 3 did not change the mobility of Band 3 during sodium dodecyl sulfate gel electrophoresis (data not show). Digestion of the carboxyl terminal of Band 3 could, however, be detected by sodium dodecyl sulfate gel electrophoresis if Band 3 was first fragmented by chymotrypsin treatment of intact cells into 60 000 and 35 000 dalton fragments, followed by endo-β-galactosidase treatment to remove the bulk of carbohydrate from the carboxyl terminal 35 000 dalton fragment. Under these conditions, the 35 000 dalton fragment migrates as a discrete band during sodium dodecyl sulfate gel electrophoresis (Ref. 26, Fig. 2), sample 3 (endo-β-treated) versus sample 4 (no endo-β-treatment).

Fig. 2 confirms that the carboxyl-terminal of Band 3 is accessible to carboxypeptidase Y in ghosts stripped of extrinsic proteins by 2 mM EDTA, pH 12.0. All carboxypeptidase Y digestions were carried out at pH 4.5. Carboxypeptidase Y digestion of cells (sample 1) or ghosts (sample 2) at pH 4.5 did not change the mobility of the 35 000 dalton carboxy-terminal fragment of Band 3 (Fig. 2). In contrast, digestion of stripped ghosts with carboxypeptidase Y at pH 4.5 produced a carboxyl-terminal fragment that migrated at a lower apparent molecular weight (sample 8, no carboxypeptidase Y versus sample 9, carboxypeptidase Y-treated). No change in the mobility of the 35 000 dalton fragment was observed if

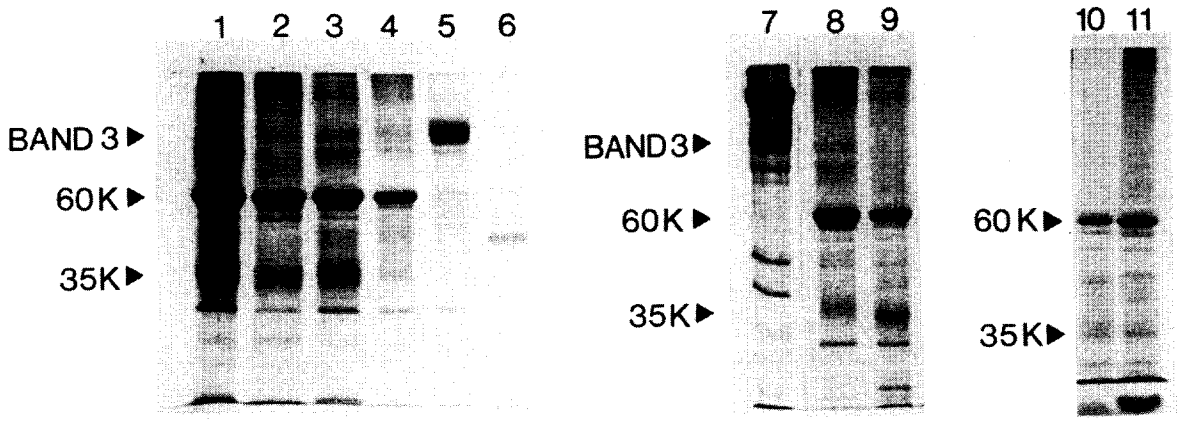

Fig. 2. Sodium dodecyl sulfate gel electrophoresis of samples containing Band 3 and its 60000 and 35000 dalton subfragments. Samples 1–4, 8–11: Cells at a 25 percent hematocrit in phosphate-buffered saline (pH 7.4) were treated with 0.5 mg/ml chymotrypsin at 37 °C for 1 h. Sample 1: Cells were treated with 5 U/ml carboxypeptidase Y at 37 °C (pH 4.5) before being treated with chymotrypsin. Ghost membranes were prepared, treated with 10 mU/ml of endo-β-galactosidase (endo B) in 10 mM sodium citrate (pH 5.7) at 37 °C for 1 h and then stripped with 2 mM EDTa (pH 12) at 0 °C to remove peripheral proteins. Sample 2: Ghosts prepared from chymotrypsinized cells were digested with 5 U/ml carboxypeptidase Y in 5 mM sodium phosphate (pH 4.5) at 37 °C for 1 h, then treated with endo B and stripped with 2 mM EDTA (pH 12) at 0 °C. Sample 3: Ghosts prepared from chymotrypsinized cells were treated with endo-β and stripped with 2 mM EDTA (pH 12) at 0 °C. Sample 4: Ghosts prepared from chymotrypsinized cells were stripped with 2 mM EDTA (pH 12) at 0 °C. Note that without endo-β treatment the 35 kDa fragment migrates as a very diffuse band. Sample 5: Purified Band 3. Sample 6: 3 μg (0.45 U) carboxypeptidase Y. Sample 7: Ghost membranes. Sample 8: Ghosts prepared from chymotrypsinized cells were digested with endo-β as described for sample 1 and stripped with 2 mM EDTA, pH 12 at 0 °C. Sample 9: As in sample 8 except that the EDTA-stripped ghosts were subsequently digested with 5 U/ml carboxypeptidase y in 5 mM sodium phosphate (pH 4.5) at 37 °C for 1 h. Sample 10: Ghosts prepared from chymotrypsinized cells were digested with endo-β as described for sample 1 and stripped with 1 M KI. Sample 11: As in sample 10, except that the KI-stripped ghosts were subsequently digested with 5 U/ml carboxypeptidase Y in 5 mM sodium phosphate (pH 4.5) at 37 °C for 1 h.

carboxypeptidase Y digestion of stripped membranes was performed at pH 5.5 or above (not shown)

It is possible that the conditions used to strip the extrinsic proteins from the ghost membrane produced a conformational change in Band 3 that resulted in exposure of an external cryptic carboxyl terminus. Any change in conformation of Band 3 must be minor or reversible since this stripping procedure did not alter the affinity of Band 3 for stilbene disulfonate inhibitors [44]. We therefore used a very different stripping procedure to remove extrinsic membrane proteins. 1 M KI at neutral pH removes extrinsic proteins as efficiently as the alkali procedure (Fig. 2, lane 10). Treatment of these stripped membranes with carboxypeptidase Y at pH 4.5 resulted in a decrease in the size of the carboxyl-terminal fragment (Fig. 2, lane 11).

No change in the mobility of the 60000 dalton fragment on sodium dodecyl sulfate gel electrophoresis was observed although the carboxyl terminal of this fragment is clearly accessible to chymotrypsin. This fragment has a carboxyl-terminal sequence -Lys-Thr-Tyr [11,24,26,27,36,38]. Carboxypeptidase Y could remove the two terminal amino acids but would not be able to remove the lysine residue under acidic conditions [34,35]. A loss of only two amino acids would not be expected to change the mobility of a 60000 dalton polypeptide on sodium sulfate gel electrophoresis significantly.

No change in the mobility of the 35000 dalton fragments was observed in intact cells or ghosts that had been treated with carboxypeptidase Y. Band 3 in membranes stripped of extrinsic proteins at alkali pH or by high salt was, however, sensitive to carboxypeptidase Y digestion at pH

4.5. These results lead to two important conclusions. Firstly, the carboxyl terminus of Band 3 is not accessible to carboxypeptidase Y in cells under conditions (pH 4.5) known to extensively digest Band 3. Secondly, these results suggest that the extrinsic proteins that are removed from the membrane by the stripping procedures may reduce access of the carboxypeptidase Y to the carboxyl terminal of Band 3. The carboxyl-terminal region of Band 3 may therefore interact with extrinsic proteins on the cytoplasmic side of the erythrocyte membrane. It is not feasible to treat intact cells at pH 12 or with 1 M KI to check for the possible exposure of the carboxyl terminus of Band 3 at the cell surface, since these treatments cause cell lysis. It is possible therefore that both stripping procedures cause an external, cryptic carboxyl terminus of Band 3 to become exposed. All the experiments described in this paper are consistent with the notion that the carboxyl terminal of the Band 3 polypeptide is located on the cytoplasmic side of the erythrocyte membrane. The evidence can be summarized as follows. Vigorous trypsin treatment of cells does not cleave Band 3 while mild trypsin treatment of ghosts produces limited cleavage of the carboxyl terminal of Band 3. Carboxypeptidase Y treatment of cells did not digest Band 3. Carboxypeptidase Y treatment of ghosts, stripped ghosts or inside-out vesicles produced only limited digestion of Band 3 unless the pH was 4.5 or less. Membranes had to be stripped of extrinsic proteins which are bound to the cytoplasmic face of the erythrocyte membrane for carboxypeptidase Y to have access to the carboxyl terminal of Band 3. The stripping procedures necessary to remove these proteins could not be applied to cells due to lysis.

A model for the folding of Band 3 in the erythrocyte membrane is presented in Fig. 3. The amino terminal of Band 3 has been localized to the cytoplasmic side of the membrane [1,2]. A hydropathy profile of the sequence of mouse Band 3 has revealed that Band 3 could cross the erythrocyte membrane up to twelve times [3,4]. Extensive digestion of Band 3 with pepsin or papain produces 4000 dalton intrinsic fragments [10,32]. These transmembrane segments are α-helical in conformation [10] and are likely arranged as an α-helical bundle consisting of polypeptide from

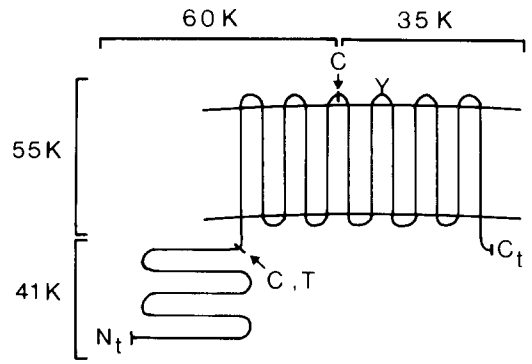

Fig. 3. Model for the arrangement of the Band 3 polypeptide in the erythrocyte membrane. C, chymotrypsin-sensitive sites; T, trypsin-sensitive site; Nt, amino terminal; Ct, carboxyl terminal, which was localized to the cytoplasmic side of the membrane in this paper; Y, carbohydrate attachment site; 41K, 41000 dalton cytoplasmic domain; 55K, 55000 dalton membrane domain, 60K, 60000 dalton amino-terminal fragment, 35K, 35000 dalton glycosylated carboxyl-terminal fragment.

two Band 3 molecules. Exterior protease cleavage sites, chemical labelling sites and the glycosylation sites define portions of Band 3 that are exposed to the outside of the erythrocyte [1–4]. The internal protease sites, chemical labelling sites, phosphorylation sites and reactive sulfhydryl groups define sites exposed to the cytoplasm of the erythrocyte [1–4]. The additional information provided in this paper localizes the carboxyl terminal of Band 3 to the cytoplasmic side of the erythrocyte membrane. Since the amino terminal of Band 3 has also been localized to the cytoplasmic side of the membrane, the Band 3 polypeptide must cross the erythrocyte membrane an even number of times. Regions of Band 3 that are exposed to the outside or inside of the cell can also be defined by using antibodies raised against synthetic antigens corresponding to sequences of interest. Such studies are at present in progress.

Acknowledgements

Anne Hansen is thanked for typing the manuscript. The Medical Research Council of Canada, The Alberta Heart Foundation and the Alberta Heritage Foundation for Medical Research provided generous financial support. One of us (R.A.F.R.) is a Scholar of the Alberta Heritage Foundation for Medical Research.

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