Isolation and Partial Characterization of Three Acidic Proteinases in Erythrocyte Membranes

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1. The distribution of proteolytic activity in membranes from human erythrocytes and from rabbit reticulocytes and erythrocytes was investigated, after removal of leucocytes and platelets from the cell suspensions. 2. All membrane preparations displayed proteolytic activity in the acidic pH region only. Membranes from human and rabbit mature erythrocytes showed latent activity, which could be increased when extracted with a number of detergents. 3. Three active fractions were resolved either by gel chromatography of solubilized membrane extracts or by standard polyacrylamide-gel electrophoresis. The three proteinase activities (designated proteinases I, II and III) were purified from solubilized extracts of human erythrocyte membranes. 4. The relevant mol.wts. were around 80000, 40000 and 30000, respectively, and each of the three proteinases appeared to be composed of a single polypeptide chain. 5. Distinctive pH optima (in the range pH 2.8–3.9) and different saturation profiles with globin as substrate were observed for proteinases I, II and III. 6. Dithioerythritol, Hg^{2+} and Cu^{2+} inhibited each of the three human enzymes, but more selective inhibitory effects were exerted by other modifiers of proteolytic enzymes and by haemin. Similar effects were observed with the three proteinases from rabbit cells. 7. The activity of the three human proteinases seems to be restricted to naturally occurring protein substrates, although with poor specificity, and none of them was active on synthetic substrates. 8. Digestion of globin by each of the three enzymes yielded similar polypeptide fragments in all cases, this indicating an endopeptidase type of activity.

In recent years proteolysis has been recognized as an important general process whereby intra- and extra-cellular events can be regulated (Goldberg & Dice, 1974). However, only limited information is available on the mechanisms of protein breakdown within human erythrocytes, in spite of the primary role that they are thought to play in determining physiological and pathological phenomena of this cell (Yoshida *et al.*, 1967; Adams *et al.*, 1972; Morelli *et al.*, 1978).

Although there is little doubt as to presence of proteolytic activity in human erythrocyte membranes (Morrison & Neurath, 1953; Moore *et al.*, 1970; Fairbanks *et al.*, 1971; Bernacki & Bosmann, 1972; Hulla, 1974; Tokés & Chambers, 1975), controversial results have emerged on the type of enzymes being associated with the membrane itself. Thus, together with reports indicating the presence of both acidic and neutral proteinases (Morrison & Neurath, 1953; Bernacki & Bosmann, 1972; Tokés & Chambers, 1975), other studies have restricted the nature of membrane proteinases to single types displaying activity either in the acidic (Hulla, 1974)

or in the neutral pH range (Moore *et al.*, 1970; Fairbanks *et al.*, 1971).

The most reasonable explanation for the heterogeneity of these findings is the presence of residual contaminating leucocytes, which appear to be the source of some membrane proteinases, and particularly of the neutral ones. This conclusion seems to be supported by the data of Reichelt *et al.* (1974), indicating the absence of any detectable neutral proteolytic activity and the presence of a single acidic proteinase in human erythrocyte membranes. This enzyme was solubilized, purified and investigated in some of its molecular and kinetic properties.

The present study was undertaken in an attempt to characterize further the nature of the proteinase activities associated with the erythrocyte membrane. Under carefully defined conditions, which are different from those reported by Reichelt *et al.* (1974), unequivocal evidence was obtained for three distinct proteinase species in both human and rabbit erythrocyte membrane. These proteinases, having optimal activity in the acidic pH range, were purified from the human membranes after removal of leucocytes and platelets from erythrocytes. The procedure of purification and some of the molecular and kinetic

Abbreviation used: Cbz, benzyloxycarbonyl.

properties of the three proteolytic enzymes are described in this paper. The results of this study demonstrate that the cathepsin-like enzymes are identifiable as endopeptidases.

Experimental

Materials

Microcrystalline cellulose (Sigmacell 50), α -cellulose and phenylhydrazine hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Triton X-100 was from Rohm and Haas, Philadelphia, PA, U.S.A., and sodium deoxycholate from Nutritional Biochemicals Co., Cleveland, OH, U.S.A. Fluorescamine was purchased from Hoffmann-LaRoche, Nutley, NJ, U.S.A. Ultrogel AC A44 was a product of LKB, Milan, Italy. DEAE-Sephadex A-50 was purchased from Pharmacia, Uppsala, Sweden. Diaflo membranes were from Amicon Corp., Lexington, MA, U.S.A. DEAE-cellulose DE32 was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. All reagents for gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Other chemicals were reagent grade. Casein, bovine serum albumin, bovine insulin, L-prolyl-L-leucylglycine, *N*-Cbz- α -L-glutamyl-L-phenylalanine, *N*-Cbz-L-glutamyl-L-tyrosine, L-leucine 2-naphthylamide, L-arginine 2-naphthylamide, L-glycyl-L-phenylalanine α -naphthylamide, α -*N*-benzoyl-DL-arginine *p*-nitroanilide, α -*N*-benzoyl-DL-arginine 2-naphthylamide, α -*N*-benzoyl-L-arginine amide, L-cysteine, iodoacetamide, *p*-chloromercuribenzoic acid, dithioerythritol, Tos-Lys-CH₂Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one hydrochloride, TLCK), Tos-Phe-CH₂Cl (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one, TPCK), leupeptin and pepstatin were purchased from Sigma. Phenylmethanesulphonyl fluoride was obtained from Cyclo Chemicals, Los Angeles, CA, U.S.A. L-Lysyl-L-alanine, L-lysylglycine and glycyl-L-leucine were obtained from Miles-Yeda, Kiriath Weizmann, Rehovot, Israel.

Methods

Preparation of erythrocytes. Erythrocytes, completely free of leucocytes and platelets, were obtained from freshly collected venous blood (either human or rabbit) essentially by the procedure described by Beutler *et al.* (1976), by using a column containing a mixture of microcrystalline cellulose and α -cellulose (1:3, w/w). Blood counts were carried out by using standard manual techniques with decreased dilution of the filtered blood cells. No leucocytes or platelets were detected by counting 10000 erythrocytes.

Preparation of reticulocytes. Reticulocytosis was

induced in adult rabbits by daily subcutaneous injections of 2.5% (w/v) phenylhydrazine hydrochloride, by the protocols of Etlinger & Goldberg (1977). The reticulocyte suspensions were then freed of leucocytes and platelets as described above for the erythrocytes.

Lysis of erythrocytes and preparation of haemoglobin-free erythrocyte membranes. Erythrocytes, prepared as above, were lysed with 10 vol. of 5 mM-sodium phosphate buffer, pH 7.5, at 4°C. The erythrocyte membranes were collected by centrifugation at 20000g for 20 min, and washed with the same buffer until the washing fluid was completely free of haemoglobin ('packed membrane pellet').

Solubilization of erythrocyte membranes. To solubilize the erythrocyte-membrane components, several procedures were used.

(a) The packed membrane pellet was resuspended in 1 vol. of 2% Triton X-100. The suspension was left for 10 min at 25°C and then sonicated in an ice bath at 1.5 A at 20000 Hz for 90 s in a 60 W MSE Ultrasonic disintegrator. The clear supernatant obtained by centrifugation for 1 h at 10000g contained approx. 70% of the total proteolytic activity recovered (i.e. the sum of the activities measured in the supernatant and in the precipitate).

(b) The packed membrane pellet was diluted with 1 vol. of a 8 mg/ml solution of sodium deoxycholate and then sonicated as described under (a). This procedure resulted in the complete solubilization of the membrane pellet and no centrifugation was therefore required.

(c) The packed membrane pellet was treated with 1 vol. of 3 M-NaCl.

(d) The packed membrane pellet was diluted with 3 vol. of water and solubilized at 25°C with butan-1-ol essentially by the procedure reported by Reichelt *et al.* (1974). The clear supernatant obtained after centrifugation for 2.5 h at 20000g was collected and concentrated by ultrafiltration through an Amicon Diaflo UM10 membrane. The solubilized erythrocyte membranes obtained by this procedure are referred to below as 'cleared butanol extract'.

Assay of proteolytic activities. The standard assay of the proteolytic activities was carried out in an incubation mixture containing various amounts of human globin (prepared as described by Hayman & Alberty, 1961) in 0.1 M-sodium citrate buffer, pH 2.8 (final volume 1.0 ml).

The incubation was carried out for 1 h at 37°C and stopped by addition of 0.2 ml of 50% (w/v) trichloroacetic acid (10% final concn.). The precipitate was discarded by centrifugation for 10 min at 2000g and the extent of proteolysis evaluated on 0.2 ml samples of the clear supernatant by using fluorescamine at pH 8.5 as developing reagent (Udenfriend *et al.*, 1972).

One unit was arbitrarily defined as the amount

of enzyme that releases 1 μ mol of free amino group/h in these conditions.

Haemin was prepared as reported by Labbe & Nishida (1957).

Protein determination. The protein concentration in the packed membrane pellet or in the cleared butanol extract was determined by the method of Lowry *et al.* (1951), by using as standard a solution of crystalline ovalbumin whose concentration was estimated from its A_{280} (Cunningham *et al.*, 1957).

In all other fractions the protein concentration was measured by alkaline hydrolysis followed by addition of fluorescamine by the procedure reported by El Dorry *et al.* (1977).

Results

Enzyme solubilization

Table 1 lists a number of treatments designed to solubilize proteolytic activity from membranes of mature human erythrocytes and from both reticulocytes and mature erythrocytes from the rabbit. The solubilized material contained proteolytic activity in the acidic pH range, but no activity could be detected at neutral pH values (see below). The patterns obtained in these experiments indicate a close similarity between the human and the rabbit erythrocyte membranes. Thus in both cases, no release of proteolytic activity into a soluble form is achieved with high-ionic-strength solutions, but marked solubilization is obtained by using detergents or butan-1-ol. The lower total activity in the butanol

extract than in the detergent-solubilized membranes does not involve inactivation, as shown by similar values of specific activity in all cases. However, the butanol extraction followed by centrifugation at 100000g ('cleared butanol extract') was routinely used for further purification, since the soluble proteins obtained by this procedure proved to be more suitable for molecular characterization.

As far as the rabbit reticulocyte membranes are concerned, their proteolytic activity, besides greatly exceeding that present in the membrane fraction of the circulating cells, is consistently less cryptic. This marked difference may reflect an age-dependent process resulting in a modified arrangement of the proteolytic system(s) within the lipid component of the membrane matrix.

Evidence for three proteinase forms in the solubilized membrane

Gel chromatography of the 'cleared butanol extract' on Ultrogel yields three well-resolved peaks of proteolytic activity with quantitative recovery (Fig. 1). Some 40% of the activity was recovered under peak I, and 24 and 36% were under peaks II and III respectively. These values were constantly observed in different experiments and by using different procedures for solubilization of the packed membrane pellet (see under 'Methods', and Table 1). Calibration of the column with standard proteins (bovine serum albumin, human haemoglobin) allowed estimation of the approximate molecular

Table 1. *Solubilization of membrane proteolytic activity by different treatments*

Packed erythrocytes, or reticulocytes, freed of leucocytes and platelets, were lysed and membrane fractions isolated and solubilized as described under 'Methods'. Values of protein and of proteolytic activity are referred to a 10ml volume of packed cells. Protein concentration was determined by using the method of Lowry *et al.* (1951). Proteolytic activity was determined at pH2.8 by using 6mg of human globin/ml as described under 'Methods'.

	Treatment	Protein (total mg)	Proteolytic activity (total units)	Specific activity (units/mg)
Human erythrocytes	Untreated	32.1	1.1	0.034
	1.5M-NaCl	32.1	1.1	0.034
	Triton X-100	30.0	10.0	0.33
	Sodium deoxycholate	32.8	10.4	0.32
	Butanol extract	16.4	5.7	0.35
	Cleared butanol extract	2.1	5.7	2.71
Rabbit erythrocytes	Untreated	45.0	2.9	0.064
	1.5M-NaCl	45.0	3.0	0.066
	Sodium deoxycholate	45.0	16.8	0.37
	Butanol extract	22.6	10.0	0.44
	Cleared butanol extract	2.5	13.0	5.2
Rabbit reticulocytes	Untreated	177.0	89.2	0.50
	1.5M-NaCl	180.0	91.0	0.50
	Sodium deoxycholate	177.0	113.8	0.64
	Butanol extract	81.5	57.1	0.70
	Cleared butanol extract	9.2	46.0	5.0

Fig. 1. Gel chromatography of the solubilized erythrocyte membrane extract

The 'packed membrane pellet' obtained from 100 ml of packed erythrocytes, freed of leucocytes and platelets, was solubilized and centrifuged to obtain the clear supernatant as described under 'Methods' ('cleared butanol extract', 50 ml). The extract was concentrated to 6 ml and then applied on to a column (3.2 cm $\times$ 150 cm) of Ultrogel AC A44, previously equilibrated with 50 mM-sodium borate buffer, pH 7.0, containing 0.5 M-NaCl. The flow rate was 0.7 ml/min; 7 ml fractions were collected and analysed for protein content (●, A_{280}) and for proteolytic activity (○) as described under 'Methods', by using human globin (2 mg/ml) as substrate. Arrows indicate the void volume (V_0) of the column (calibrated with Blue Dextran 2000, from Pharmacia, Uppsala, Sweden) and the elution volumes of bovine serum albumin (BSA), dimeric human haemoglobin [Hb($\alpha\beta$)] (prepared as described by Chiancone *et al.*, 1970) and Bromophenol Blue (bb). Blue Dextran, Bromophenol Blue and the two standard proteins were run together, each at a concentration of 6 mg/ml of 50 mM-sodium borate buffer, pH 7.0, containing 0.5 M-NaCl.

weights of the three proteinase fractions, as above 80 000, about 40 000 and about 30 000, respectively.

Since the above findings are at variance with those reported by Reichelt *et al.* (1974), which were consistent with a single proteinase in the solubilized membranes, the 'cleared butanol extract' was fractionated exactly by the procedure followed by these authors, i.e. by chromatography on DEAE-Sephadex. A single peak of proteinase activity was eluted in these conditions, which accounted for nearly 50% of the original activity. Further gel chromatography of this peak on Ultrogel separated the activity into three distinct fractions centred at the same elution volumes as those obtained by direct gel chromatography of the solubilized membrane extract.

Identical patterns were observed by fractionating on Ultrogel the solubilized membrane extracts from rabbit reticulocytes and erythrocytes. Thus, with the

Fig. 2. Polyacrylamide-gel electrophoresis of the solubilized erythrocyte membrane extract

A fraction of the concentrated 'cleared butanol extract' from erythrocyte membranes, prepared as described in the legend to Fig. 1 and containing 0.8 mg of protein, was applied to a 1.1 mm-thick polyacrylamide slab gel (5%, pH 8.9), and the electrophoresis was performed at 100 V for 2 h at 4°C (Maizel, 1971). After electrophoresis the gel was cut perpendicularly to the direction of migration into several slices each 5 mm thick and the content of these was separately eluted with 1.0 ml of 0.15 M-NaCl, containing 20 mM-sodium phosphate buffer, pH 7.0, for 24 h, and dialysed against water for 6 h. Proteolytic activity was tested on each fraction by the procedure described under 'Methods' with human globin (2 mg/ml) as substrate.

reticulocyte membrane, three peaks of proteolytic activity can be obtained containing 28, 51 and 21%, respectively, of the activity units layered on the column. For membrane extracts from mature erythrocytes, the three peaks had a slightly different distribution of the activity, i.e. 45, 50 and 5%, respectively.

In order to check by different experimental approaches the presence of different species of proteinases within the human erythrocyte membrane, the 'cleared butanol extract' was submitted to slab gel electrophoresis. The gel was then cut into slices whose content was eluted and assayed for enzyme activity. As Fig. 2 shows, three clearly resolved bands were obtained containing proteolytic activity, this supporting the view of three distinct proteinases in the erythrocyte membrane.

Partial purification of the three proteinase activities

This was performed starting from solubilized

Table 2. DEAE-cellulose chromatography of the three proteolytic enzymes solubilized from human erythrocyte membranes and previously separated by gel chromatography

The three protein peaks containing the proteolytic activity and obtained by gel chromatography on an Ultrogel AC A44 column, as described in the legend to Fig. 1, were separately collected and concentrated to 0.01–0.1 mg of protein/ml. Proteinase I was dialysed against 0.1 M-sodium acetate, pH 5.0; proteinases II and III were dialysed against 0.1 M-sodium borate, pH 7.0. Each of the three proteinases was then separately applied to a different column (12cm × 1 cm) of DEAE-cellulose DE 32 equilibrated with the same buffer used for the dialysis. Proteinase I was eluted with a linear NaCl gradient from zero to 1 M in 0.1 M-sodium acetate, pH 5.0 (total volume of the gradient, 54ml). The flow rate was 0.3 ml/min and 3ml fractions were collected. The overall recovery of proteolytic activity was 70%. Proteinase II was eluted by 0.1 M-sodium citrate, pH 3.5, with an 80% recovery of activity. Proteinase III was eluted with a linear NaCl gradient from zero to 1 M in 0.1 M-sodium borate, pH 7.0 (total volume of the gradient, 56ml).

Proteinase	Adsorbed at pH	Eluted by	Specific activity (units/mg of protein)	Purification factor
I	5	0.2M-NaCl	2.24	2.0
II	7	pH 3.5	100.0	2.8
III	7	0.4M-NaCl	49.0	1.3

membrane extracts of human erythrocytes. After gel chromatography on Ultrogel resulting in fractionation of the original proteolytic activity into three peaks (Fig. 1), each of these was individually collected, concentrated to a 2 ml volume and chromatographed on a column of DEAE-cellulose. In order to achieve a better purification of the three proteinase fractions,

the conditions used in the anion-exchange step were modified from those followed by Reichelt *et al.* (1974) for the DEAE-Sephadex chromatography and resulting in a single peak of proteolytic activity. As shown in Table 2, distinctive conditions of adsorption and elution apply to each proteolytic activity, this representing a further argument for the occurrence of three proteinase forms. These are designated below as proteinases I, II and III.

Each of the three proteinases purified by gel chromatography and DEAE-cellulose was submitted to slab gel electrophoresis. The resulting electrophoretic pattern is not dissimilar from that recorded with the solubilized membrane extract (shown in Fig. 2). Comparison between the migration of proteolytic activity and that of protein shows the presence of two contaminating bands in proteinase III, whereas proteinase II appears to be electrophoretically homogeneous (Fig. 3).

In order to estimate the molecular size of the three membrane proteinases under dissociating conditions, slab gel electrophoresis was combined with electrophoretic analysis in the presence of sodium dodecyl sulphate. For this purpose, after the three partially

Fig. 3. Standard polyacrylamide-gel electrophoresis of the partially purified proteinases

Samples of each proteinase partially purified by DEAE-cellulose chromatography (see Table 2) and containing 0.05 mg of protein were subjected in duplicate to slab polyacrylamide-gel electrophoresis in the conditions reported in the legend to Fig. 2. After electrophoresis one of the duplicate tracks was stained with Coomassie Blue and the other excised. The content of each slice was eluted and assayed for proteolytic activity as described in the legend to Fig. 2. (a), Proteinase I; (b), proteinase II; (c), proteinase III.

purified proteinases had been run separately on a standard gel as in the experiment shown in Fig. 3, each portion of the gels containing the proteolytic activities was excised. The corresponding contents were eluted, concentrated and submitted to polyacrylamide-gel slab electrophoresis in the presence of sodium dodecyl sulphate and β -mercaptoethanol. Standard proteins of known molecular weight (bovine serum albumin, muscle fructose 1,6-bisphosphate aldolase, human globin) were run in parallel. The results reported in Fig. 4 show apparent homogeneity of the three proteinases, whose molecular weights under dissociating conditions are approx. 80000 (I), 40000 (II) and 30000 (III). Comparison of these values with those obtained by gel filtration suggests that each of the three membrane proteinases is a single polypeptide chain of different length.

Fig. 4. Slab polyacrylamide-gel electrophoresis of the three proteinases under dissociating conditions

The three distinct proteolytic enzymes purified by slab-gel electrophoresis as described in Fig. 3 were eluted from the gel (see the legend to Fig. 2), concentrated by vacuum dialysis and analysed by slab polyacrylamide (5%)-gel electrophoresis in sodium dodecyl sulphate at pH 7.0, in the presence of β -mercaptoethanol, under the conditions described by Weber & Osborn (1969). Proteins were stained with Coomassie Blue. Known molecular-weight standards (bovine serum albumin, rabbit muscle fructose 1,6-bisphosphate aldolase, human globin) were run in separate lanes under the same experimental conditions. Their migration is indicated by arrows in the left margin. Each protein was prepared for electrophoresis by suspending it in a solution containing (final concns.) 10 mM-sodium phosphate, pH 7.0, β -mercaptoethanol (3%, v/v), sodium dodecyl sulphate (2%, w/v), glycerol (5%, v/v). The samples were heated at 100°C for 2 min and immediately applied to the gel: 50 μ l containing approx. 50 μ g of protein was loaded per gel.

Fig. 5. Effect of pH on the proteolytic activity present in the cleared butanol extract of human erythrocyte membranes

The proteolytic activity was assayed as described under 'Methods', with human globin (6 mg/ml of incubation mixture) as substrate. The buffers were the following: pH 1.5–4.0, 0.1 M-sodium citrate; pH 4.0–6.0, 0.1 M-sodium acetate. The pH values reported in the graph were the actual ones measured in each final incubation mixture.

pH optimum and substrate-saturation curves

As shown in Fig. 5, the pH curve of the overall proteolytic activity present in the crude solubilized membrane extract from human erythrocytes reveals two major peaks centred around pH 2.8 and pH 3.9, respectively. No appreciable proteinase activity can be detected in the neutral region of pH. Similar pH profiles were observed when human globin was replaced with other acid-denatured protein substrates or when proteolytic activity from the packed membrane pellet was instead solubilized with Triton X-100 or sodium deoxycholate. Failure to observe neutral activity on globin persisted also when 5 mM- Ca^{2+} and the erythrocyte cytoplasm were added to the membrane pellet, according to the experimental conditions of King & Morrison (1977).

In the experiments aimed at exploring the pH-dependence of each of the three human proteinases, the combined effects produced by varying the substrate concentration were also investigated. Preliminary experiments indicated optimal activity in the acidic range between pH 2.0 and 4.5, although the pH optimum appeared to be somewhat distinctive for each of the three proteinase species.

The differences among the three proteolytic enzymes with respect to pH optimum and saturation profile are shown in Fig. 6. Thus the proteolytic activity displayed by proteinase I at pH 3.9 is higher than at pH 2.8, with a pH 3.9/pH 2.8 activity ratio of 1.47. An apparent K_m value for human globin of

Fig. 6. Proteolytic activity of the three proteinases at pH 2.8 and 3.9 as a function of the substrate concentration. The three proteinases were obtained by gel chromatography on Ultrogel of the cleared butanol extract, as reported in the legend to Fig. 1. The proteolytic activity of each proteinase was measured at pH 2.8 (●) and at pH 3.9 (○), by using increasing concentrations of human globin, under the conditions reported under 'Methods'. (a), Proteinase I; (b), proteinase II; (c), proteinase III.

Table 3. Effect of metal ions and EDTA on the three membrane proteinases from human erythrocytes. Samples of the three proteinases obtained as described in the legend to Fig. 1, corresponding to approx. 0.3 unit, were preincubated in 0.7 ml with 0.05 M-sodium borate, pH 7.5, in the presence of the various cations at the concentrations indicated, for 10 min at 37°C. Then 0.2 ml of 0.5 M-sodium acetate, pH 3.9 (proteinases I and III), or of 0.5 M-sodium citrate, pH 2.8 (proteinase II), and 0.1 ml of globin (20 mg/ml) were added to each preincubation mixture. The pH was adjusted for each incubation to values of 3.9 (proteinases I and II) and 2.8 (proteinase II) by dropwise addition of 1 M-HCl. Zn^{2+} and Cu^{2+} were used in the sulphate form; all other cations were in the chloride form.

Cation	Concn. (mm)	Residual activity (%)		
		Proteinase I	Proteinase II	Proteinase III
None		100	100	100
Ca^{2+}	10	100	100	100
Mg^{2+}	10	100	100	100
Zn^{2+}	10	100	75	100
Cu^{2+}	5	0	44	10
Hg^{2+}	5	13	11	27
EDTA	10	100	100	100

0.05 mg/ml was estimated at both pH values. Inspection of Fig. 6(a) reveals a progressive inhibition by substrate appearing from 0.4 mg/ml onwards. A clearly distinctive situation applies to proteinase II, whose pH optimum is 2.8, with an apparent K_m value of 0.038 mg/ml. The pH 3.9/pH 2.8 activity ratio is 0.5 (Fig. 6b). There is negligible inhibition by high substrate concentrations. The pH-dependence of the proteinase activity contained in peak III is not dissimilar from that of peak I, except for a higher K_m value (0.18 mg/ml) and for different K_m values at pH 3.9 and 2.8. The pH 3.9/pH 2.8 activity ratio is 1.2 (Fig. 6c).

The three proteinases prepared from membranes

of rabbit cells showed properties nearly identical with those of human enzymes as regards pH optimum and affinity for globin (both human and rabbit globin were used). This was found with mature erythrocytes and with almost homogeneous populations of reticulocytes. Thus, for instance, the pH 3.9/pH 2.8 activity ratios were as follows for the three proteinases obtained from reticulocyte membrane: 2.1 (I), 0.42 (II) and 1.5 (III).

Effects of various ions and EDTA

Of the metal ions tested, only Hg^{2+} and Cu^{2+} at high concentrations had an inhibitory effect which was comparable for all of the three proteinases pre-

Table 4. *Influence of thiol reagents and of various modifiers on the catalytic activity of the three membrane proteinases from human erythrocytes*

For experimental conditions see the legend to Table 3. Repeated additions of 1 mM-phenylmethanesulphonyl fluoride were made during the incubations, in view of the short half-life of this compound in aqueous solutions.

Modifier	Concn. (mM)	Residual activity (%)		
		Proteinase I	Proteinase II	Proteinase III
None		100	100	100
<i>p</i> -Chloromercuribenzoic acid	1	80	100	100
Iodoacetamide	1	100	100	70
2-Mercaptoethanol	10	100	90	65
L-Cysteine	1	100	72	35
	5	100	16	5
Dithioerythritol	1	76	76	50
	5	26	26	11
Tos-Phe-CH ₂ Cl	1	100	100	25
Tos-Lys-CH ₂ Cl	1	100	100	80
Phenylmethanesulphonyl fluoride	1	100	100	100
Haemin	0.0125	100	100	60
	0.0625	100	100	52
	0.125	100	100	35
Leupeptin	5 µg/ml	100	71	50
	10 µg/ml	100	28	17
Pepstatin	0.1 µg/ml	100	0	20
	1 µg/ml	90	0	11

Table 5. *Hydrolysis of different protein substrates by the three acid proteinases*

All proteins were denatured by exposure for 10 min at pH 1.8 (in HCl), followed by extensive dialysis against water. Each protein was used at a final concentration of 2 mg/ml. The three proteinases, recovered by filtration on Ultrogel as described in Fig. 1, were concentrated to 0.01–0.1 mg of protein/ml. For each incubation mixture samples of each proteinase corresponding to approx. 0.3 unit were used. The incubation was carried out as described under 'Methods' by using 0.1 M-sodium acetate buffer, pH 3.9 (proteinases I and III), and 0.1 M-sodium citrate buffer, pH 2.8 (proteinase II).

Substrate	Hydrolysis (% of that of globin)		
	Proteinase I	Proteinase II	Proteinase III
Human globin	100	100	100
Human haemoglobin	100	100	54
Bovine insulin	100	100	100
Casein (Hammarsten)	18	13	20
Bovine albumin	10	77	20

pared from human erythrocytes. EDTA was completely ineffective (Table 3).

Thiols and other inhibitors

Table 4 shows the influence of a number of thiols and of known modifiers of proteolytic enzymes on the activity of the proteinases obtained from human erythrocyte membranes. Dithioerythritol has a marked inhibitory effect on all of the three enzymes, but some distinctive effects are exerted by the other thiols, and especially by cysteine, which is strongly inhibitory to proteinases II and III, but not to proteinase I.

Among other modifiers of proteolytic enzymes,

Tos-Phe-CH₂Cl, Tos-Lys-CH₂Cl, phenylmethanesulphonyl fluoride, leupeptin and pepstatin were ineffective on proteinase I. In contrast, the activity associated with proteinase II was inhibited by pepstatin and leupeptin. Finally, the activity of proteinase III, besides being extremely sensitive to both pepstatin and leupeptin, was also lowered by the addition of reagents known to inactivate serine proteinases.

The most specific and selective inhibitory effect seems to be that produced by haemin, which is effective only toward the activity of proteinase III. Such sensitivity to the haemoglobin prosthetic group observed by using globin (haem-deprived) as the protein substrate explains the different rate of

hydrolysis of globin compared with acid-denatured haemoglobin, which applies to proteinase III only (see Table 5).

The most effective of the modifiers listed in Table 4 were also tested on the three acidic proteinases prepared from membrane of rabbit cells. Again, the three enzymes from both circulating (adult) erythrocytes and reticulocytes were examined with respect to this property. The distinctive inhibitory effects on each of the three proteinases appear to be maintained also with the rabbit enzymes. This is particularly the case with pepstatin and haemin, which afford the most selective effects among the modifiers used.

Proteolytic activity on other protein substrates

The extent of hydrolysis of several protein substrates is reported in Table 5. Various acid-denatured proteins from a number of sources appear to be susceptible to degradation, although to a different extent, by each of the three proteinases prepared from human erythrocyte membranes. Neither the crude extract nor the three separated proteinases showed hydrolysing activity towards any of the synthetic substrates of cathepsin A (*N*-Cbz-L-glutamyl-L-tyrosine, *N*-Cbz-L-glutamyl-L-phenylalanine), cathepsin B (α -*N*-benzoyl-DL-arginine *p*-nitroanilide, α -*N*-benzoyl-DL-arginine 2-naphthylamide, α -*N*-benzoyl-L-arginine amide) and cathepsin C (L-glycyl-L-phenylalanine 2-naphthylamide). The same was true for the following compounds: L-prolyl-

L-leucylglycine, L-leucine 2-naphthylamide, L-lysyl-L-alanine, L-lysylglycine, L-glycyl-L-leucine.

Time course and extent of digestion of human globin by the three proteinases from human erythrocyte membranes

Fig. 7 shows the products arising from digestion of human globin by each of the three acidic proteinases. Inspection of the patterns of globin breakdown reveals that the hydrolytic activity associated with the three enzymes is of an endopeptidase type. Furthermore, no gross differences are apparent in the number and molecular size of the polypeptide fragments produced by each of the three proteinases. Thus, in all cases, the initial protein subunit (16000 mol.wt.) is cleaved to discrete species with mol.wts. of 13000, 11000 and below 10000.

Discussion

The results of the present investigation indicate that three different endopeptidases account for the proteolytic activity detectable in solubilized erythrocyte membranes. This conclusion is especially supported (a) by the isolation of three acidic proteinases, irrespective of the procedure followed (i.e. gel chromatography or polyacrylamide-gel electrophoresis), (b) by the identical results obtained starting from human and rabbit erythrocytes and from rabbit reticulocytes, and (c) by the distinctive patterns of pH-dependence, substrate-saturation curves, substrate specificity and response to a number of modifiers.

Although the clearly different molecular weights represent an additional argument in favour of the existence of three proteinases, the available data cannot discriminate between the possibility of three discrete proteins with different primary structures and that of multiple enzyme subforms arising from a common precursor or through interconversion. The results reported in the present study do not allow a precise classification of the three cathepsin-like enzymes to be made. However, the demonstration of an array of proteolytic enzymes, rather than of a single proteinase, within the membrane, seems to introduce complexity in the mechanisms of protein breakdown within the erythrocyte. Among the questions raised by such multiplicity of proteinases, a major one is that of defining specific roles for each proteinase in terms of protein substrates, accessibility of soluble proteins to their site of degradation, selective mechanisms of regulation etc. Thus, for instance, Quirk *et al.* (1978) have reported that band 3, the major protein of erythrocyte membranes, may be a substrate for a specific proteinase activity in erythrocytes induced to fuse by treatment with oleoylglycerol. A possibly related system has been

Fig. 7. Patterns of globin digestion by the three human erythrocyte membrane proteinases

Proteinases I, II and III were incubated as reported in the legend to Fig. 1 with 2 mg of globin/ml, by using 0.4 unit of each proteinase. At the time indicated, samples were removed from the incubation mixtures, the reaction was stopped with trichloroacetic acid (10%, final concn.) and the extent of globin hydrolysis was analysed by slab polyacrylamide (10%) gel electrophoresis in sodium dodecyl sulphate under the conditions reported in the legend to Fig. 4. Ribonuclease A and cytochrome *c* (both from Sigma) were used as standard proteins of known molecular weight.

described by King & Morrison (1977), who have attributed some significant changes in membrane proteins to a Ca^{2+} -activated proteinase. None of the three acidic proteolytic enzymes described here appears, however, to be identifiable with such a Ca^{2+} -activated proteinase, which requires the presence of cytoplasmic proteins to display activity at neutral pH (King & Morrison, 1977).

Another type of correlation between soluble and membrane-bound proteolytic enzymes could be afforded by haemin. In fact, this powerful and selective inhibitor of proteinase III is also able to decrease the activity of the ATP-dependent proteolytic system described by Etlinger & Goldberg (1977) in the soluble fraction of reticulocytes and thought to be responsible for the intracellular degradation of abnormal proteins, including mutant haemoglobins.

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