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PROTEOLYTIC ACTIVITY ASSOCIATED WITH HUMAN ERYTHROCYTE MEMBRANES

SELF-DIGESTION OF ISOLATED HUMAN ERYTHROCYTE MEMBRANES

ZOLTÁN A. TÖKÉS* and SUSAN M. CHAMBERS

Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel (Switzerland)

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SUMMARY

At least two kinds of enzymes are active in the proteolytic self-digestion of erythrocyte membranes. The specific activities of these enzymes do not decrease with repeated washings of purified stroma. The effects of a variety of inhibitors on the membrane preparation's capacity to digest ^{125}I-labelled casein, covalently linked to latex beads, have been examined.

Pepstatin-inhibitable enzyme, active at low pH, digests the membrane extensively to small polypeptide fragments. Spectrin, located at the internal part of the membrane, is readily degraded. Diisopropylfluorophosphate-inhibitable enzyme, active at pH 8–9, has only limited digestive capacity. Some of the membrane components, such as the small molecular weight glycoproteins, are resistant to digestion. The restricted capacity of digestion is due to the membrane molecular arrangement; increased disaggregation removes the restriction and increases the activity. Spectrin is not digested unless the membrane topography is disrupted by NP-40 neutral detergent. These observations suggest that the enzymes active at basic pH are located external to the cell. Intact cells do possess a limited capacity to degrade ^{125}I-labelled casein when their surfaces are brought into contact with substrate-coated beads.

INTRODUCTION

Membrane-associated proteases have recently received increased attention in a wide variety of biological systems, for example in the bacterial membrane of *Escherichia coli* [1], in their possible relationship to contact inhibition in viral transformed eukaryotic cells [2, 3] and in connection with the stability of membrane immunoglobulin receptors [4].

* To whom all communication should be addressed. Current address: LAC/USC Cancer Center, Research Bldg. One, Rm 252, School of Medicine, University of Southern California, 1720 Zonal Avenue, Los Angeles, Calif. 90033, U.S.A.

Abbreviations: TLCK, Nα-Tosyl-L-lysylchloromethane hydrochloride; PMSF, phenylmethylsulfonylfluoride.

Erythrocyte membranes were studied by Morrison and Neurath [5] who first reported the presence of two kinds of proteolytic activity with pH 3 and pH 8 optima in a crude stroma suspension. They used hemoglobin and casein as substrates and assayed for the activity by a change in trichloroacetic acid precipitability. The neutral protease activity extracted by 0.75 M potassium thiocyanate showed marked activation by reducing agents and a complete inactivation by mercuric ions. Soya bean trypsin inhibitor was ineffective. Both enzyme activities were recovered by *n*-butanol extraction, but this time the neutral protease was not activated by reducing agents or inactivated by mercuric ions suggesting that several enzymes with neutral or slightly basic pH optima are present in the membrane. Moore et al. [6] followed up the original observations and reported additional information on extraction and substrate specificity. Natural substrates: stroma, stroma sialoglycoprotein, plasma Cohn Fraction I, casein and hemoglobin were tested. The assay was terminated by addition of trichloroacetic acid and digestion was measured by a change in precipitability. No activity was found at pH 7.4 with plasma Cohn Fraction I or with synthetic trypsin-specific substrates. Sialo-proteins and stroma were degraded. Ultracentrifugation studies on a Sephadex G-100 fraction from stroma extract, containing proteolytic activity, suggested that the activity was associated with lipoproteins. Bernacki and Bosmann [7] extracted human erythrocyte plasma membranes with 0.1 % Triton X-100 neutral detergent and assayed for proteolytic activity by a change in trichloroacetic acid precipitability of ^{3}H-labelled acetylated hemoglobin. In this detergent extract they found that cathepsin-D like activity was 75 times greater than the enzyme activity at pH 7.4.

We have studied the role of proteolytic enzymes in self-digestion of erythrocyte membranes. In attempts to isolate membrane components, we found significant degradation of some membrane proteins only under certain experimental conditions. In addition, some of the degradations were very restricted and failed to result in an alteration of trichloroacetic acid precipitability. We therefore used a modified protease assay to avoid trichloroacetic acid precipitation. In this method, ^{125}I-labelled substrates were covalently linked to plastic beads. The rate of release of radioactivity gives a very sensitive indication of proteolytic activity. In addition we applied extraordinary care to remove platelets and leukocytes since their enzyme content is several orders of magnitude higher than that of erythrocytes. In this paper we present the results of experiments on enzyme levels in erythrocyte membranes and the possible mode of action of membrane-associated enzymes in self-digestion under various disaggregation and pH conditions.

MATERIALS AND METHODS

Erythrocyte Preparation

Fresh human venous blood was heparinised and macrophages, granulocytes, some of the lymphocytes and platelets were adsorbed onto cotton wool or nylon fiber. Erythrocytes and a fraction of lymphocytes were washed off with Hanks' medium. This cell suspension was layered over 8.25 % Ficoll (Pharmacia, Uppsala, Sweden), specific gravity 1.077, and centrifuged at $1000 \times g$ for 20 min. Erythrocytes packed in the Ficoll layer were collected and washed with phosphate-buffered saline,

pH 7.4, and the fractionation through Ficoll repeated. Cells were washed free of Ficoll and stroma prepared immediately.

Stroma Preparation

Cells were lysed in 20 mosM phosphate buffer, pH 7.4, containing 10^{-4} M $CaCl_2$ for 30 min at 0–4 °C. Stroma was collected by centrifugation at 30 000 × *g* for 45 min, washed four times and resuspended in 310 mosM phosphate buffer. A second osmotic lysis was effected by a 20-fold dilution with cold glass-distilled water and followed by two additional washings with 20 mosM phosphate buffer with or without calcium. Aliquots were dissolved in 1 % sodium dodecylsulfate and the absorbance spectrum measured. If traces of hemoglobin were still detected a third osmotic lysis, followed by two more washes, was necessary. 1.0 *A* at 280 nm corresponds to 0.6–0.7 mg stroma protein per ml, as determined by the method of Lowry et al. [8]. The pelleted stroma so obtained was either lyophilised or kept at −70 °C.

Protease Activity

This was determined by a new method developed in our laboratory (Tökés, Z. A., Chambers, S. M. and Kiefer, H., unpublished), using ^{125}I-labelled protein substrates covalently linked to modified latex beads, 25 μm diameter, with carbodiimide. The modified polystyrene latex beads, (Bio-Bifunctionals, P.O. Box 54591, Los Angeles, Calif. 90054, U.S.A.) carry amino groups on their surface. Coupling was carried out using 4×10^7 beads in 1.5 ml of 0.1 M phosphate buffer, pH 7.2, and 50–100 μg of previously ^{125}I-labelled and extensively dialysed casein in 0.5 ml buffer. 4 mg of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (Bio-Bifunctionals) was added and the coupling reaction was carried out for 40 min at room temperature. The beads were extensively washed and stored at 0 °C. The rate of ^{125}I released from these beads is proportional to the protease activity. All incubations were carried out under sterile conditions in the presence of 10 000 units/ml penicillin, and 10 000 μg/ml streptomycin, (Grand Island Biochemical Co., New York, U.S.A.). Controls used 1 % human serum albumin, electrophoretically pure (Behringwerke AG, Marburg-Lahn, W. Germany) with or without varying concentrations of non-ionic detergent NP-40 (Shell Oil Co., Zürich, Switzerland). Protease activity available on the intact erythrocyte surface was estimated by incubating cells with ^{125}I-labelled protein coupled to latex and measuring the delocalisation of radioactivity.

To estimate the effect of proteolysis on the membrane components, different concentrations of stroma preparations were incubated under sterile conditions with penicillin and streptomycin for varying lengths of time. This form of self-digestion was terminated by the addition of 2.3 % sodium dodecylsulfate sample buffer, and all samples were heated to 100 °C for 7 min. Sodium dodecylsulfate-polyacrylamide gel electrophoresis was carried out according to Laemmli [9] on 10 % polyacrylamide gels, which were stained for protein using Coomassie brilliant blue or for carbohydrate with periodic acid - Schiff reagent according to Fairbanks et al. [10]. The absorbance of the stained gels was monitored on a Gilford 2400 spectrophotometer. Human immunoglobulin, IgG, heavy and light polypeptide chains, ovalbumin and chymotrypsinogen were used as molecular weight markers.

Protease Inhibitors

$N\alpha$-Tosyl-L-lysylchloromethane hydrochloride (TLCK) was obtained from E. Merck A. G., Darmstadt, W. Germany. Trasylol (Bayer, Leverkusen, W. Germany) was purified from sodium-2 (ethylmercurithio)-benzoate by pressure dialysis. Tritiated diisopropylfluorophosphate, 5 Ci/g, was obtained from The Radiochemical Centre, Amersham, U.K. Pepstatin was a gift of Dr Umezawa, Banyu Pharmaceutical Co., Osaka, Japan. Phenylmethylsulfonylfluoride (PMSF) was obtained from Calbiochem, San Diego, Calif. 92112, U.S.A.

Sonication was carried out with Elgasonic Instrument, Elga SA, Bienne, Switzerland, using 25 kHz for various lengths of time.

Stroma labelled with ^{3}H-labelled diisopropylfluorophosphate was electrophoresed on 10 % polyacrylamide gels in 1 % sodium dodecylsulfate and the gels were sliced into 2-mm sections, treated with Protosol tissue solubilizer (Nuclear Chicago, Des Plaines, Ill. 60018, U.S.A.) overnight at 37 °C and the radioactivity measured with 35–40 % efficiency in a Packard Tri-Carb liquid scintillation spectrometer. The presence of diisopropylphosphate, a breakdown product of diisopropylfluorophosphate interfered with the mobilities in sodium dodecylsulfate gel electrophoresis. This was eliminated by extensive lyophilisation over a trap containing NaOH.

RESULTS

Erythrocyte lysates contain numerous peptidases. Therefore, it is necessary to thoroughly wash the stroma preparation. The complete absence of hemoglobin is used as an indication of successful washing. We were not able to achieve this with less than two osmotic lyses and seven washes. Only such preparations gave constant proteolytic activity per A unit at 280 nm. Fig. 1 shows the effect of stroma washing on the specific proteolytic activity at pH 3.4 and pH 8.6. Repeated washing did not alter the specific activity. If the stroma was washed without the addition of Ca^{2+} to the phosphate buffer, a lower level of enzyme activity was obtained at both pH, but repeated washing did not reduce the specific activities. On this basis, we describe the enzyme activity as "membrane associated".

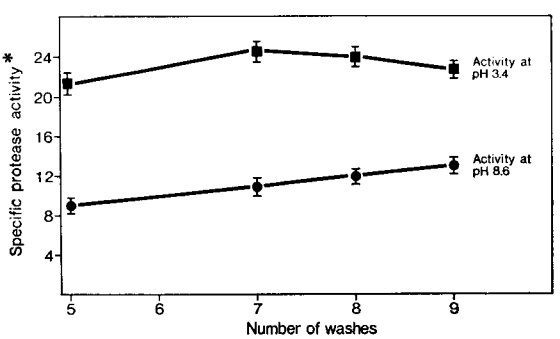

Fig. 1. The effect of repeated washing on the specific protease activity of stroma. The activity is expressed as percent of ^{125}I-labelled casein substrate digested by stroma diluted to 4 A/ml at 280 nm. 150 μl stroma suspension was incubated with ^{125}I-labelled casein, $2 \cdot 10^4$ cpm, coupled to latex beads suspended in 20 μl of phosphate buffered saline at pH 8.6 or in pH 3.4 citrate/phosphate buffer.

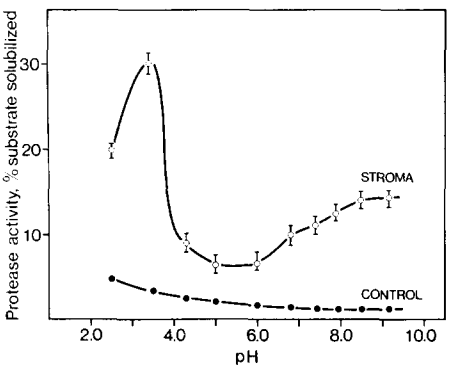

Fig. 2. The pH dependence of protease activity from human erythrocyte stroma. Packed stroma was diluted to 5 *A* per ml at 280 nm and NP-40 was added to a final concentration of 0.02 %. 20 μl of diluted stroma was mixed with 150 μl of appropriate buffer and presented to the ^{125}I-labelled casein substrate, $2 \cdot 10^4$ cpm, covalently linked to modified latex beads. Activity was determined by measuring the percentage of ^{125}I released after 5 h incubation under sterile conditions at 37 °C. Results are the average of three separate experiments.

The pH dependence of protease activity from human erythrocyte stroma is shown in Fig. 2. The results are similar to those reported by Morrison and Neurath [5] from a crude stroma suspension. The reproducible shoulder on the activity curve between pH 6.5 and 9.0 suggests the presence of more than one protease. The ratio of enzyme activity in acidic to basic pH varied from one experiment to another, between 2 : 1 and 5 : 1, but in no experiment did we find 75 times greater activity at pH 3.4 as reported by Bernacki and Bosmann [7]. The relative activities varied with different iodinated protein substrates; human immunoglobulin and horse heart cytochrome *c* were relatively resistent to digestion at neutral pH.

In order to find out whether any proteolytic activity is available at neutral pH on the surface of intact cells, we incubated 10^7 erythrocytes with 5×10^4 latex beads to which 2×10^4 cpm ^{125}I-labelled casein had been covalently linked. The incubation was carried out in phosphate-buffered saline, pH 7.6, at 37 °C for 3 h. To estimate the amount of proteolytic activity released, supernatant was collected from equal numbers of cells preincubated for the same length of time and presented to ^{125}I-labelled casein beads in a similar way to the cells. 1.8 % of substrate was digested by 40 ng trypsin under the same conditions (Table I). The activity of the supernatant

TABLE I

PROTEOLYTIC ACTIVITY OF THE INTACT ERYTHROCYTES

^{125}I-labelled casein, $2 \cdot 10^4$ cpm, linked to latex beads in 20 μl phosphate-buffered saline, pH 7.5, was incubated with 100 μl sample for 3 h at 37 °C. Erythrocytes were gently brought into contact with the beads by centrifugation for 2 min at 500 rev./min.

Sample	^{125}I-labelled casein released (%)
40 ng trypsin	1.80
10^7 erythrocytes	2.81
Supernatant from 10^7 erythrocytes	1.99

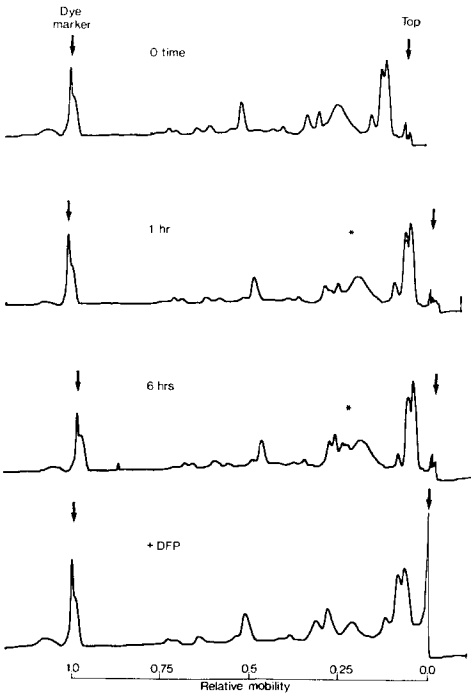

Fig. 3. Self-digestion of erythrocyte stroma at pH 8. Stroma was incubated for 0, 1 and 5 h and subjected to disc electrophoresis in sodium dodecylsulfate. The gels were stained with Coomassie blue and scanned at 520 nm. The top trace is the control without any incubation, and the bottom one shows stroma which was incubated in the presence of diisopropylfluorophosphate. Asterisks indicate the main region of modification.

probably comes from enzymes absorbed to the cell surface and released during pre-incubation or from the few cells that might have lysed during handling. The activity directly attributable to the presence of cells is 0.82 % which corresponds to 17.8 ng trypsin equivalent activity per 10^7 cells.

Stroma prepared by washing seven times, and adjusted to pH 8, was incubated for varying periods of time at 37 °C. As indicated in Fig. 3, only very minor changes were observed even after 5 h of incubation. These changes were mainly confined to membrane components with a molecular weight between 90 000 and 60 000. Only trace amounts of small molecular weight polypeptides appeared, indicating that the cleavage was very restricted. The addition of 20 mM diisopropylfluorophosphate inhibited the degradation. Even if the stroma was vesicularised by sonication the extent of degradation did not increase. Spectrin, the major large molecular weight membrane component, was not affected by digestion for 8 h. If the incubation mixture contained 10^{-4} M Ca^{2+}, the extent of the membrane self-digestion was greater but qualitatively indistinguishable.

The extent of digestion at pH 3 is far greater than at pH 9 (Fig. 4). The degradation appears to affect all membrane protein components. Spectrin, located on the inner surface of the membrane, is extensively degraded. As judged from the de-

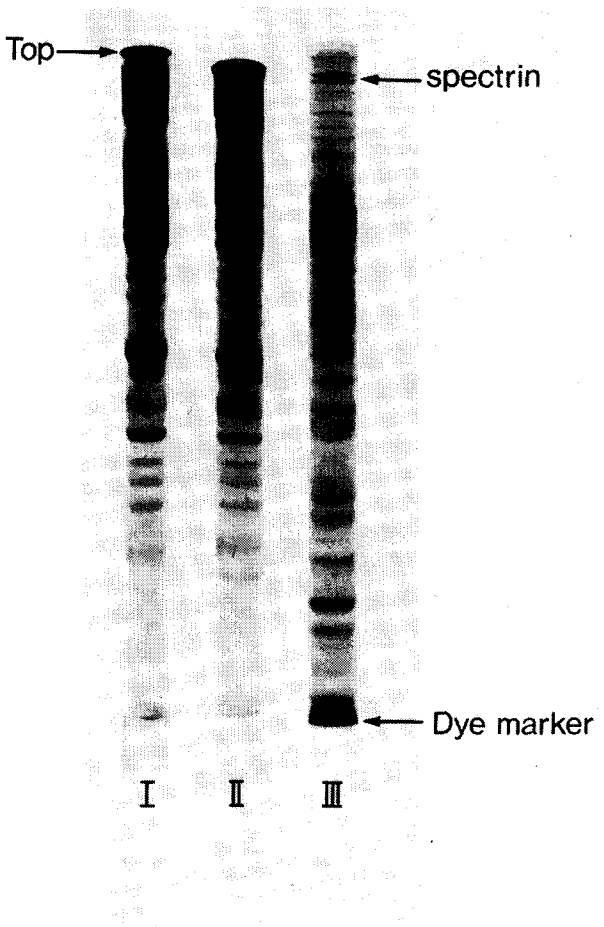

Fig. 4. Self-digestion of erythrocyte stroma as a function of pH. Stroma was prepared as described, and immediately mixed with sodium dodecylsulfate buffer in sample I. The pH was adjusted to 9, in sample II, and to 3 in sample III. Samples were incubated for 5 h at 37 °C, dissolved in sodium dodecylsulfate containing sample buffer, and heated to 100 °C for 5 min. Equal amounts were applied to 10 % polyacrylamide disc gels and electrophoresed. The gels were stained for proteins with Coomassie blue.

crease of absorbance in the spectrin double band, more than 85 % of the protein has been degraded to lower molecular weight components during the low pH incubation. The appearance of low molecular weight polypeptides indicates that the digestion is extensive and not restricted to a limited degradation as with digestion at pH 9.

The effect of self-digestion at pH 8 on the membrane glycoproteins is illustrated in Fig. 5. Three major bands were found, identified as glycoproteins 1, 2 and 3; periodic acid-Schiff reagent stained periodic acid Schiff I, II, III according to Fairbanks et al. [10]. Their relative amounts varied slightly from one stroma preparation to another. Under all incubation and pH conditions used, glycoprotein 3 remained unchanged as judged by sodium dodecylsulfate gel electrophoresis. Both glycoprotein 1, $R_f = 0.207$ and 2, $R_f = 0.508$, slightly decreased after 5 h incubation and new

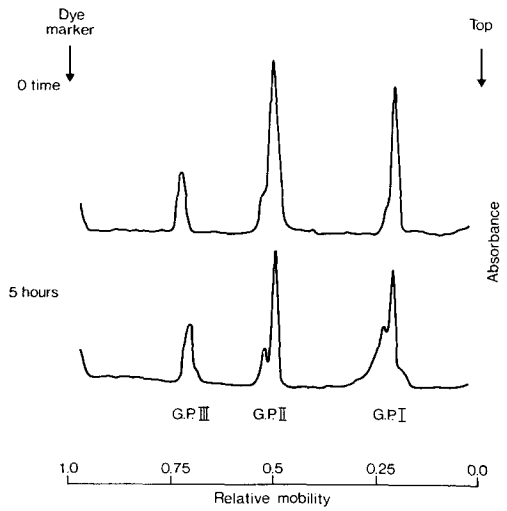

Fig. 5. Self-digestion of erythrocyte stroma and its effect on membrane glycoproteins. Stroma was incubated in phosphate-buffered saline pH 7.9 in the presence of 10^{-4} M Ca^{2+}, penicillin and streptomycin. Equal amounts of samples, before and after 5 h of incubation at 37 °C, were electrophoresed in sodium dodecylsulfate and the gels were stained for carbohydrate with periodic acid-Schiff reagent. The scanning of the gels at 560 nm is shown above.

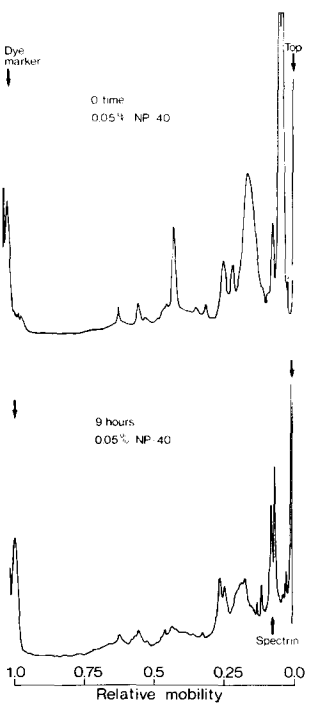

Fig. 6. Self-digestion of erythrocyte stroma in the presence of NP-40 detergent. Stroma was disrupted with 0.05 % NP-40 in phosphate-buffered saline pH 7.9, incubated for 9 h at 37 °C and subjected to electrophoresis in sodium dodecylsulfate. The absorption scan of the gels stained with Coomassie blue is shown. The upper trace is the control sample. Identical amounts of protein were applied to each disc gel.

species with $R_f = 0.223$ and $R_f = 0.538$ appeared, respectively, migrating as though the molecular weight had decreased by 4000. This conversion was not complete even after 15 h of incubation.

The addition of non-ionic detergent increased the proteolytic activity, as judged by both the extent of self-digestion and the proteolytic enzyme assay. Fig. 6

TABLE II

THE EFFECT OF VARIOUS ADDITIVES ON THE PROTEOLYTIC ACTIVITY OF ERYTHROCYTE MEMBRANES

All incubations were carried out for 3 h at pH 8.0, except for the low pH enzyme digestion, with and without pepstatin, where the pH was 3.4. $2 \cdot 10^4$ cpm ^{125}I-labelled casein coupled to latex beads in 100 μl buffer were incubated with 50 μl stroma suspension, previously sonicated; 4 A at 280 nm, equivalent to 125 μg stroma protein per sample. Inhibitors were added to the stroma 30 min prior to incubation. Controls always included 1 % human serum albumin in addition to the inhibitors. All experiments were performed three times, each time in duplicate. Under these experimental conditions 0.1 μg trypsin releases 4.4 % of the radioactivity, 880 cpm.

Samples	Additives	cpm released	Inhibition (%)
Control, pH 3.4	1 mM pepstatin	1300	—
Stroma, pH 3.4	none	3900	—
Stroma, pH 3.4	1 mM pepstatin	1400	96
Control	20 mM diisopropylfluorophosphate	130	—
Stroma	none	1450	—
Stroma	10 mM diisopropylfluorophosphate	720	55
Stroma	20 mM diisopropylfluorophosphate	600	64
Control	5 mM Zn^{2+}	150	—
Stroma	none	1200	—
Stroma	1 mM Zn^{2+}	900	29
Stroma	5 mM Zn^{2+}	680	50
Control	40 mM EDTA	150	—
Stroma	none	1600	—
Stroma	40 mM EDTA	650	66
Control	1 mM glutathione	540	—
Stroma	none	1700	—
Stroma	1 mM glutathione	1650	4
Control	1 mg/ml Trasylol	200	—
Stroma	none	1200	—
Stroma	1 mg/ml Trasylol	600	60
Control	10 mM PMSF	150	—
Stroma	none	1500	—
Stroma	5 mM PMSF	950	41
Stroma	10 mM PMSF	800	52
Control	40 mM TLCK	300	—
Stroma	none	1800	—
Stroma	40 mM TLCK	400	93
Control	0.02 % NP-40	500	—
Stroma	none	1600	—
Stroma	0.02 % NP-40	1900	27 increase

shows that in the presence of a concentration of 0.05 % NP-40 the degradation is far more extensive affecting the large molecular weight membrane protein, spectrin, also. The detergent disaggregation of the membrane, however, failed to increase the degradation of glycoproteins.

The effects of various inhibitors on the enzyme activity are summarized in Table II. More than 90 % of the proteolytic activity at pH 3.4 was inhibited by 1 mM pepstatin; it had no effect on neutral proteases. 20 mM diisopropylfluorophosphate blocked 60–70 % of the activity at pH 8. Under these conditions acetylcholinesterase

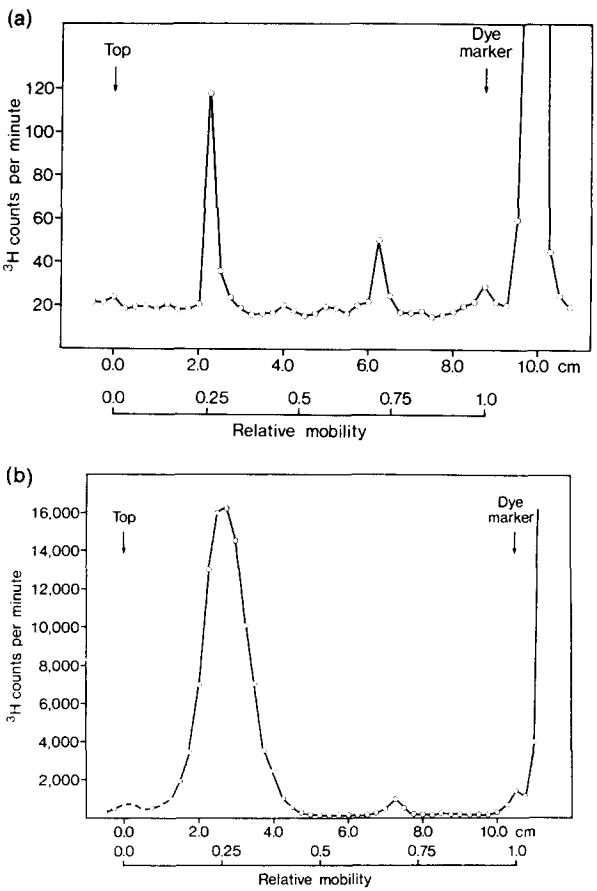

Fig. 7. (a) Sodium dodecylsulfate gel electrophoresis of erythrocytes labelled with ^{3}H-labelled diisopropylfluorophosphate. $5 \cdot 10^6$ human erythrocytes in 100 μl of phosphate-buffered saline, pH 7.9. were incubated with 20 μl of ^{3}H-labelled diisopropylfluorophosphate diluted 10-fold with buffer. 2 μCi equivalent. The cells were incubated for 15 min at 4 °C and for 15 min at 37 °C. The labelled cells were washed three times with phosphate-buffered saline, pH 7.4, resuspended in 20 μl buffer and dissolved in sodium dodecylsulfate sample buffer, heated to 100 °C for 5 min and applied to disc gels for electrophoresis. The front peak, off scale, is the breakdown product of diisopropylfluorophosphate, diisopropylphosphate. (b) Sodium dodecylsulfate gel electrophoresis of erythrocyte stroma labelled with ^{3}H-labelled diisopropylfluorophosphate. Stroma from approx. $150 \cdot 10^6$ cells was prepared as described and incubated with 15 μCi ^{3}H-labelled d-isopropylfluorophosphate in phosphate-buffered saline, pH 7.9, for 20 min at 37 °C. An equal volume of sodium dodecylsulfate sample buffer was added, the whole sample heated to 100 °C for 5 min and electrophoresed.

activity was more than 95 % inhibited. Increasing the diisopropylfluorophosphate concentration 5-fold completely inhibited the activity. This need not be due to the covalent linkage to serine in an enzyme active site; diisopropylphosphate, an inactive breakdown product of diisopropylfluorophosphate, also inhibits at this concentration. Zn^{2+} did not activate the enzyme activity, as reported earlier [5], and at 5 mM concentration significantly reduced the activity. Similarly reduced glutathione and β-mercaptoethanol, not included in the table, had no effect on the activity. 40 mM TLCK eliminated more than 90 % of the activity. PMSF and EDTA were less efficient inhibitors. EDTA in less than 5 mM concentrations had no effect. Purified Trasylol inhibited 60 % of the activity at a concentration of 1 mg/ml. The neutral protease activity was consistently and significantly enhanced by as little as 0.02 % NP-40. Optimum concentrations for maximum effect of the detergent cannot be established by the method used; membrane proteins become increasingly available for digestion and compete for the substrate, casein.

Bellhorn et al. [11] have shown that diisopropylfluorophosphate specifically binds acetylcholinesterase in the erythrocyte membrane. We subjected ^{3}H-labelled diisopropylfluorophosphate-treated erythrocytes to sodium dodecylsulfate gel electrophoresis. In addition to the 85 000–90 000 molecular weight protein band, identified as acetylcholinesterase by Bellhorn et al. [11], a protein migrating as a 25 000 $\pm$ 3000 molecular weight component showed radioactive incorporation; this is shown in Fig. 7a. When erythrocyte membranes were prepared and reacted with ^{3}H-labelled diisopropylfluorophosphate (Fig. 7b), the relative amount of the 85 000 molecular weight component was greater, indicating that some of the serine-containing active sites of acetylcholinesterase were not available on intact cells. This, however, was not found with the 25 000 molecular weight component. A total of 65 cpm per 25 000 molecular weight band was observed for 5×10^6 erythrocytes after labelling of stroma or intact cells. Assuming a 100 % efficiency in labelling, specific activity of 660 Ci/M of ^{3}H-labelled diisopropylfluorophosphate and 40 % efficiency in counting disintegrations per min, this would amount to 13 000 molecules per cell. At present this component is being investigated for protease activity as a most likely candidate, since all other diisopropylfluorophosphate binding proteins are present in quantities of less than 1000 molecules per cell membrane.

DISCUSSION

We define enzymes as membrane associated if their specific activity per mg protein first increases and then remains constant with substantial purification of the membrane. In this definition the molecular nature of association can vary from ionic or hydrophobic interaction to an actual immersion in the bilayer and a lipid requirement for enzyme activity. Although both the acidic and basic enzyme activities meet these criteria for being membrane associated, the molecular nature of this relationship has not been thoroughly investigated. Burkholder and Brecher [12] studied the interaction between proteolytic enzymes and bovine erythrocyte membranes, and reported selective binding and inhibition of proteases, particularly of the blood coagulation system. They have reported that totally acetylated trypsin has reduced binding capacity to ghosts, which indicates that some of the proteases may be adsorbed by the formation of ionic bonds. Morrison and Neurath [5] identified two neutral proteases.

Erythrocyte proteinase I was obtained by extraction of crude stroma suspension with potassium thiocyanate. This enzyme may have been associated with the membrane through ionic bonds and, unlike erythrocyte proteinase II, it is activated by reducing agents such as ascorbic acid and sulfhydryl compounds. Both enzymes were recovered in the aqueous layers after *n*-butanol extraction of crude stroma suspension. It was postulated that *n*-butanol cleaves lipid-protein complexes [5]. Such a complex was also suggested by Moore et al. [6] based on ultracentrifugation studies.

Proteolytic activity between pH 6.5 and 9 is compatible with the presence of two enzymes, one with a pH optimum around 7.4 and another one with higher pH optimum. The ratio of acidic to basic protease activity varies with different procedures for membrane preparation. Extensive erythrocyte purification and repeated lysis for ghost preparation could easily explain the difference between our results and those reported earlier by Bernacki and Bosmann [7]. The low pH activity is inhibitable by pepstatin. This pentapeptide blocks specifically cathepsin-D and other related acid proteinases [13]. Cathepsin-D is widely distributed in lysosomes [14], and lymphocytes are particularly rich in this enzyme [15]. Therefore, it is conceivable that the erythrocyte acidic protease is of lysosomal origin. Spectrin, located internally to the membrane [16], was rapidly degraded at pH 3.4 indicating availability of the enzyme to spectrin. This observation, however, has to take into account that the membrane preparation denatures under these pH conditions, disrupting most of the membrane molecular arrangement.

As illustrated in Fig. 4, the degradation at pH 3 is extensive, resulting in numerous smaller polypeptides. This extensive degradation is in part due to greater activity at this pH, and also due to enzyme action on a denatured complex, which has lost its molecular order. At pH 8, the protease action is still restricted to a few molecular species. With progressive dissociation a proteolytic activity emerges. This release of activity could be attributed either to the presence of an inhibitor in the membrane or to a molecular arrangement which would not allow contact between a potential substrate and the enzyme. Since ^{3}H-labelled diisopropylfluorophosphate is capable of labelling the enzyme on the intact cells or in the undissociated stroma, the active site must be available. The restriction of activity might best be explained by limited freedom of the enzyme within the membrane. Neutral detergents, like NP-40, disrupt the two-dimensional array and thereby make even spectrin available for digestion. If the neutral protease activity originates from the outer segment of the membrane, topological restriction would make spectrin unavailable for proteolysis.

Some enzymatic activity was detected at the surface of intact erythrocytes. Cells brought in contact with ^{125}I-labelled casein coupled to beads, released more radioactivity than their corresponding supernatants. This digestive capacity, however, cannot be directly compared with trypsin activity, for this enzyme was in solution and not adsorbed to a cell surface. Some of the erythrocyte surface enzymes may also be lost during the seven washes for membrane preparation. This surface activity was not detected in the presence of serum, diluted 10-fold. It is not clear whether this is due to the presence of inhibitors in serum or to competition by excess substrate. The activity may also be unique to some protein substrates for ^{125}I-labelled human immunoglobulins were not attacked. Indeed casein, a phosphorylated protein, may be a special substrate, perturbing the membrane to such an extent that normally inhibited proteolytic activity is revealed as a consequence of encounter.

Change in molecular weight of glycoproteins may not be entirely due to proteolysis. Bosmann [17] reported the presence of 10 glycosidic enzymes in human erythrocyte plasma membranes, of which nine were thought to be on the external surface. These enzymes, which had an optimum pH between 4.2 and 5.0, liberated carbohydrate from intact cells. However, in our experiments using sodium dodecylsulfate-polyacrylamide gel electrophoresis, minor changes in carbohydrate composition of glycoproteins would go undetected. Sequential elimination would result in a gradual decrease in apparent molecular weight. The alteration we observe creates a new and distinct entity, as if a defined fragment is removed. Since diisopropylfluorophosphate inhibits this process, an enzyme with serine in the active site is inferred, but this does not prove the protease character of the enzyme. Even prolonged self digestion for 15 h at 37 °C failed to convert completely glycoprotein I and glycoprotein II into the smaller molecular weight component. This might be due to a molecular heterogeneity of the glycoproteins and different sensitivity to proteolysis. Indeed Tanner and Boxer [19] have been able to purify glycoproteins free of the major sialoglycoprotein and reported unique amino acid and carbohydrate composition. The striking similarity by which both glycoprotein I and II components were degraded would indicate a molecular relationship. Such a suggestion was reported recently by Marton and Garvin [18].

It is interesting to note that under all conditions of digestion between pH 7 and 9, with or without neutral detergent, glycoprotein III remained unaffected as judged by sodium dodecylsulfate electrophoresis. This glycoprotein is not a breakdown product produced during the membrane purification for it is present in intact, untreated cells disrupted in sodium dodecylsulfate at elevated temperatures. Such a membrane component could serve as a protein inhibitor by virtue of its resistance.

Although the additives, listed in Table II, may not act directly on the enzymes responsible for proteolysis, observations on inhibitors are worthy of comment. In order to minimize any degradation of membrane components during fractionation, we recommend a buffer around pH 6, since the lowest activity was observed here. In addition, pepstatin, diisopropylfluorophosphate, and TLCK should be included in the medium from the time of cell disruption. Although there is evidence that diisopropylfluorophosphate covalently links to some of the membrane proteins, not all the inhibition is due to blocking serine in the active sites of enzymes. We have repeatedly noted inhibition by diisopropylphosphate as well. ^{3}H-labelled diisopropylphosphate binds to the membrane and is not removed by repeated washings, as judged by its presence in sodium dodecylsulfate gel electrophoresis of the stroma from labelled cells. Its presence in the membrane could perturb the molecular topography resulting in secondary inhibitory effects. The same caution applies to all other inhibitors studied. Enzymes separated from the phospholipid membrane components may behave differently in the presence of Zn^{2+} and glutathione, and could even be activated as reported earlier [5]. Similarly, Trasylol, a polypeptide containing 58 amino acids, could be a more efficient inhibitor of a purified enzyme in solution than when the enzyme is trapped in a membrane complex. A similar argument also applies to the NP-40-dependent increase in activity. This detergent could free the enzymes from the surface and make the substrate more available to their active sites. It could also dissociate enzyme-inhibitor complexes present in the membrane; however, there is only suggestive evidence for the existence of such complexes [12].

Partial inhibition both by PMSF and d-isopropylfluorophosphate, even in large excess, proves the existence of more than one proteolytic enzyme. Both of these inhibitors covalently link to a serine residue in the enzyme active site. Such enzymes are not known to be inhibited by Zn^{2+} or EDTA at concentration used in these experiments. This would imply that a second enzyme with a different active site is responsible for the diisopropylfluorophosphate-uninhibitable activity. About 93 % of the activity is inhibited by TLCK, a specific irreversible inhibitor with a high affinity of binding to the active site of trypsin. There is, however, no evidence that the inhibition in the erythrocyte membrane is through the same molecular mechanism as in the case of trypsin.

^{3}H-labelled diisopropylfluorophosphate binding proteins are candidates for the neutral protease activity. If the major diisopropylfluorophosphate binding protein, with 85 000 molecular weight is the cholinesterase enzyme, the 25 000 molecular weight protein is the most likely candidate. 13 000 molecules per cell represent the lowest estimate, since a 100 % efficiency in labelling was assumed. Experiments are in progress to establish the identity of this component.

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