Purification of a protease in red blood cells that degrades oxidatively damaged haemoglobin

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Haemoglobin damaged by exposure of red blood cells to oxidants is rapidly degraded by a proteolytic pathway which does not require ATP [Fagan, Waxman & Goldberg (1986) *J. Biol. Chem.* **261**, 5705–5713]. By fractionating erythrocyte lysates, we have purified two proteases which hydrolyse oxidatively damaged haemoglobin (Ox-Hb). One protease hydrolysed small fluorogenic substrates in addition to Ox-Hb. Its molecular mass was ~ 700 kDa and it consisted of several subunits ranging in size from 22 to 30 kDa. This enzyme may be related to the high-molecular-mass multicatalytic proteinase previously isolated from a variety of tissue and cell types. The other Ox-Hb-degrading activity had an apparent molecular mass of 400 kDa on gel filtration, a subunit size of 110 kDa and an isoelectric point between 4.5 and 5.0. This protease also hydrolysed the small polypeptides insulin and glucagon, as well as other large proteins such as lysozyme. Insulin blocked the degradation of Ox-Hb and Ox-Hb blocked the hydrolysis of insulin by the purified protease. Thiol reagents and metal chelators strongly inhibited the hydrolysis of both Ox-Hb and insulin, whereas inhibitors of serine, aspartic and thiol proteases had little effect. These properties suggest that the Ox-Hb-degrading activity purified from rabbit erythrocytes is the cytosolic insulin-degrading enzyme that is believed to play a role in the metabolism of insulin in several tissues. We propose that this enzyme may also function as a key component in a cytoplasmic degradative pathway responsible for removing proteins damaged by oxidants.

INTRODUCTION

All cells must have mechanisms to prevent the accumulation of abnormal proteins which might arise as the result of mutation, biosynthetic error, denaturation or post-synthetic modification such as oxidation. In mammalian cells the breakdown of short-lived proteins and of abnormal proteins produced by the incorporation of amino acid analogues is believed to be carried out by a non-lysosomal energy-requiring proteolytic pathway [1]. This process involves the ATP-dependent ligation of ubiquitin to the amino groups of proteins and requires the participation of several protein factors [1]. Proteins to which ubiquitin is covalently attached are then susceptible to hydrolysis by a high-molecular-mass ATP-dependent protease [2,3].

Treatment of intact reticulocytes with oxidants such as phenylhydrazine [4,5], xanthine/xanthine oxidase [6,7] or hydrogen peroxide [6–8] greatly enhances protein breakdown. Similar observations have been made (a) with intact erythrocytes [7,9] that have lost one or more components of the ATP-dependent proteolytic pathway with maturation [10], and (b) with intact reticulocytes and erythrocytes depleted of ATP [9]. Dialysed reticulocyte and erythrocyte extracts also rapidly degrade purified haemoglobin treated with phenylhydrazine in the absence of ATP [9]. Therefore the breakdown of oxidant-treated haemoglobin (Ox-Hb) does not require energy, in contrast to the breakdown of endogenous reticulocyte proteins [9] or other types of abnormal proteins [1].

The ATP-independent degradation of Ox-Hb in erythrocytes does not require the participation of membrane components [11], and therefore must be primarily cytoplasmic, since erythrocytes do not contain lysosomes [12]. Inhibitors of the various classes of

proteases have been used in intact red blood cells as well as in crude extracts to better define the properties of the enzymes that degrade Ox-Hb. We and others have shown that the breakdown of proteins damaged by oxidants is sensitive to cysteine-modifying reagents and metal-chelating agents, but not to inhibitors of aspartic proteases ([11,13]; J. M. Fagan & L. Waxman, unpublished work). Inhibitors of serine proteases had no effect on the degradation of Ox-Hb in red cell lysates [9], but were reported to block the breakdown of BSA damaged by exposure to various oxygen free radicals [13].

The role of several red blood cell proteases in the degradation of oxidatively damaged proteins was also investigated. By means of specific inhibitors we showed that the calpains and thiol proteases such as cathepsin B do not participate in the hydrolysis of Ox-Hb [11]. The removal of the 700 kDa multicatalytic proteinase by immunoprecipitation did not affect the ability of extracts to degrade Ox-Hb ([14]; J. M. Fagan & L. Waxman, unpublished work), nor did preparation of extracts in 20% glycerol [11], a condition shown to maintain this proteinase in an inactive or latent state [15]. The di-isopropylfluorophosphate (DFP)-sensitive proline endopeptidase present in red blood cells [16] is also not likely to degrade Ox-Hb, since the enzymes that hydrolyse Ox-Hb appear to be insensitive to DFP [9] and other inhibitors of serine proteases ([9]; J. M. Fagan & L. Waxman, unpublished work).

In this paper we describe the purification of an enzyme from rabbit erythrocytes which appears to play a major role in the breakdown of oxidant-treated haemoglobin and show that it is identical to an insulin-degrading protease described previously [17,18].

Abbreviations used: Ox-Hb, oxidant-treated haemoglobin; DFP, di-isopropylfluorophosphate; DTT, dithiothreitol; Me₂SO, dimethyl sulphoxide; AMC, amidomethylcoumarin; MNA, methoxynaphthylamine; PEG, poly(ethylene glycol); Z-, benzylloxycarbonyl-; NEM, *N*-ethylmaleimide; PCMBs, *p*-chloromercuribenzenesulphonate; PMSF, phenylmethanesulphonyl fluoride; DCIC, 3,4-dichloroisocoumarin; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Ep-475, *trans*-epoxysuccinyl-L-leucylamido-(3-methyl)butane; TGF- α , transforming growth factor- α ; suc-, succinyl-.

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EXPERIMENTAL

Chemicals

Reagents, proteins, peptide fluorogenic substrates and protease inhibitors were purchased from Enzyme Systems Products, Sigma, and Cambridge Research Biochemicals. 3,4-Dichloroisocoumarin (DCIC) was purchased from Boehringer and Ep-475 was from Taisho Pharmaceutical. DEAE-cellulose (DE-52) was obtained from Whatman. Pentyl-agarose and Polybuffer 74 were from Sigma. Columns for f.p.l.c. were from Pharmacia and LKB. Poly(ethylene glycol) 8000 (PEG) was obtained from VWR and ammonium sulphate was from Schwarz-Mann. Rabbit erythrocytes were obtained from Pel-Freez and were used within 24 h of bleeding of the animals. Haemoglobin was purified from rabbit erythrocytes as described previously [5].

Assays of protein breakdown

Haemoglobin, insulin and casein were radiolabelled by reductive methylation [19] using [^3H]NaBH₄ (New England Nuclear) and formaldehyde. ^{125}I -labelled glucagon, insulin and BSA were purchased from New England Nuclear. BSA was denatured by reduction and alkylation in guanidine [20]. [^3H]Haemoglobin was damaged by treatment with 20 mM-phenylhydrazine [3] at pH 9.0 in 50 mM-sodium borate. The breakdown of these substrates by partially purified enzymes or by column fractions was determined as described previously [21], and specific details are given in the text and Table legends.

Hydrolysis of fluorogenic peptides

The cleavage of peptide fluorogenic substrates was measured using procedures described previously [22], with minor modifications. In a reaction volume of 300 μl were included 50 mM-Tris/HCl (pH 8.0), 10 mM-MgCl₂, 0.1 mM-dithiothreitol (DTT), 150 μM peptide dissolved in dimethyl sulfoxide (Me₂SO) (final concentration of Me₂SO in the assay was 5%) and samples (25–50 μl) of selected column fractions. After 20 min at 37 °C, peptide hydrolysis was terminated by the addition of 100 μl of 1% SDS and 1.7 ml of sodium borate (0.2 M, pH 9.1). Fluorescence was measured at 380 nm excitation and 460 nm emission for peptide–amidomethylcoumarin (AMC) substrates and at 335 nm excitation and 410 nm emission for peptide–methoxynaphthylamine (MNA) substrates.

Other methods

Protein was quantified by the method of Lowry *et al.* [23] or the dye-binding assay of Bradford [24] with BSA as the standard. PAGE in the presence of SDS was carried out according to Laemmli [25] on 10% or 12.5% acrylamide slab gels. The proteins were stained with Coomassie Blue or with silver with a kit obtained from ICN. Molecular mass standards were from Sigma.

Enzyme purification

Rabbit blood (2.5 litres) was washed with 0.9% NaCl to remove plasma proteins and white blood cells. This step and all subsequent ones in the purification were carried out at 4 °C. The resulting 1 litre of packed red blood cells was lysed by the addition of 2 vol. of ice-cold water containing 1 mM-DTT, and the mixture was stirred for 30 min. Membranes were removed by centrifugation (30 min at 20000 g) and the supernatant was decanted and the pH adjusted with 1 M-Tris base to 7.0. The membrane-free extract was supplemented with glycerol to a concentration of 20% (v/v) to maintain the multicatalytic

proteinase in an inactive (or latent) state [15]. The extract was applied to a 600 ml column of DEAE-cellulose (5 cm $\times$ 32 cm) equilibrated in a buffer containing 20 mM-Tris/HCl (pH 7.0), 20% glycerol and 0.1 mM-DTT. The column was washed with this buffer until the haemoglobin was removed, and the bound proteins were eluted with a gradient of NaCl up to a final concentration of 0.5 M. A broad peak of Ox-Hb-degrading activity was found which was eluted between 25 mM- and 300 mM-NaCl (results not shown). Since little purification was obtained by gradient elution, this activity was eluted with 2.5 vol. of 0.3 M-NaCl in subsequent purifications.

The active material was concentrated by precipitation with PEG. An aqueous solution of 50% (w/v) PEG was added slowly (over 30 min) with stirring to a final concentration of 10%, and the mixture was stirred for an additional 30 min. The precipitated protein was removed by centrifugation (20 min at 20000 g), and the pellet (which contained approx. 40% of the total protein and less than 25% of the Ox-Hb-degrading activity from the DEAE-cellulose elution) was discarded. PEG was added to the supernatant in a similar fashion to a final concentration of 20%. Following centrifugation, the protein pellet was dissolved in 50 ml of 20 mM-Tris/HCl (pH 7.0) buffer containing 10% glycerol and 0.1 mM-DTT. The 10–20% PEG step precipitated more than 75% of the Ox-Hb-degrading activity and only 16% of the protein which was eluted from the DEAE-cellulose column. The subsequent steps are described in the Results section.

RESULTS

The enzymes that degrade Ox-Hb bind to DEAE-cellulose

When a membrane-free extract of rabbit erythrocytes is applied to DEAE-cellulose at neutral pH, haemoglobin, the major protein in these cells, fails to bind and is found in the flow-through [26]. A typical experiment is shown in Table 1, in which the proteins necessary to degrade Ox-Hb are quantitatively retained by DEAE-cellulose and can be eluted with 0.5 M-NaCl. The flow-through has negligible activity by itself, and only slightly (4–14%) but reproducibly enhances proteolysis when reconstituted with the proteins eluted with high salt concentrations. Similar results were obtained whether proteolysis was measured by the production of acid-soluble fragments from [^3H]haemoglobin treated with phenylhydrazine or by the release of free amino acids from this substrate. Since additional chromatographic steps will remove the aminopeptidases necessary to generate free amino acids from peptide fragments, the formation of radiolabelled fragments from Ox- ^3H Hb was used to monitor the purification of enzymes which carry out the initial cleavages in this substrate.

Purification of the protease that degrades Ox-Hb

Hydroxyapatite chromatography. Following batch elution of the Ox-Hb-degrading activity (see the Experimental section) from DEAE-cellulose, the proteins that were precipitated between 10 and 20% PEG were applied directly to a column of hydroxyapatite and eluted with a gradient of potassium phosphate up to 0.3 M. The Ox-Hb-degrading activity bound weakly to hydroxyapatite and was completely eluted by 100 mM-phosphate (Fig. 1a). The multicatalytic proteinase, detected using the fluorogenic substrate Z-Leu-Leu-Glu-MNA [27], bound tightly to hydroxyapatite and was eluted between 0.1 and 0.2 M-phosphate (Fig. 1a; [15]).

In preliminary experiments, the Ox-Hb-degrading activity obtained after DEAE-cellulose chromatography was concentrated with ammonium sulphate instead of PEG. Ammonium sulphate was added to a final concentration of 80% (w/v), and the precipitate collected by centrifugation was

Table 1. Hydrolysis of Ox-Hb by erythrocyte lysate after DEAE-cellulose chromatography

Dialysed erythrocyte lysate (50 μ l), DEAE flow-through (50 μ l) or protein which bound to the DEAE column and was eluted with 2 vol. of 0.5 M-NaCl (100 μ l) was incubated with 325 μ g of Ox-[3 H]Hb (625000 d.p.m.) in buffer containing 50 mM-Tris/HCl (pH 8), 5 mM-MgCl₂ and 0.5 mM-DTT. After 1 h at 37 °C, the reaction was stopped by the addition of trichloroacetic acid (7% final concentration). Assays were done in triplicate and acid-soluble radioactivity and the release of alanine were measured in the same assay tube.

	Hydrolysis of Ox-[3 H]Hb to:	
	Acid-soluble fragments (μ g/h)	Amino acids (nmol of Ala/h)
Erythrocyte lysate	5.97	5.84
DEAE flow-through	0	0.13
DEAE eluate (0.5 M-NaCl)	5.67	4.93
DEAE flow-through + DEAE eluate (0.5 M NaCl)	5.93	5.62

Fig. 1. Hydroxyapatite chromatography of the Ox-Hb-degrading activity after ammonium sulphate or PEG precipitation of the DEAE eluate

The 0.3 M-NaCl DEAE eluate precipitated with either 10–20% PEG (a) or 40–80% ammonium sulphate (b) was applied to a column of hydroxyapatite (5 cm $\times$ 8 cm). Bound proteins were eluted with a linear gradient (total 500 ml) of 0.005–0.3 M-KH₂PO₄ (pH 7.1; $\times$). Fractions [1.5 ml (b) or 3 ml (a)] were collected and 25 μ l was assayed for the degradation of Ox-Hb (■), determined as described in Table 1) and for the hydrolysis of Z-Leu-Leu-Glu-MNA (□). The peak of fluorogenic peptide-degrading activity was eluted at approx. 0.15 M-KH₂PO₄ in both (a and b) hydroxyapatite runs.

redissolved and applied to the hydroxyapatite column. As shown in Fig. 1(b), the major portion of the Ox-Hb-degrading activity was eluted in the same position as the activity that hydrolyses Z-Leu-Leu-Glu-MNA. By comparing this hydroxyapatite profile

Fig. 2. Fractionation of the Ox-Hb- and insulin-degrading activity on pentyl-agarose

The hydroxyapatite (PEG) column fractions that hydrolysed Ox-Hb were pooled, precipitated with 40–80% ammonium sulphate, resuspended in 45% ammonium sulphate and applied to a pentyl-agarose column (2.5 cm $\times$ 10 cm). After washing the column with buffer containing 30% ammonium sulphate, bound proteins were eluted with a linear gradient (total 400 ml) of increasing glycerol (0–30%) and decreasing ammonium sulphate (30–0%). Ox-Hb degradation (■) was assayed as described in Table 1. Insulin hydrolysis (□) was similarly determined, except that 2 μ g of [125 I]-insulin (150000 c.p.m.) was added, and the assays were stopped by the addition of trichloroacetic acid (15% final concentration).

to that obtained after PEG precipitation, it can be concluded that ammonium sulphate activates an enzyme that hydrolyses Ox-Hb. One likely candidate is the multicatalytic proteinase. Activation of this enzyme by freezing the erythrocyte lysate in the absence of glycerol also stimulated the degradation of Ox-Hb ([11]; J. M. Fagan & L. Waxman, unpublished work). Consequently, ammonium sulphate was not used in the purification until the multicatalytic proteinase could be removed by hydroxyapatite chromatography (Fig. 1a). However, we have shown that the multicatalytic proteinase can be immunoprecipitated from lysates with no effect on the rate of degradation of Ox-Hb (J. M. Fagan & L. Waxman, unpublished work). Therefore the multicatalytic proteinase probably is not involved in degrading Ox-Hb.

Pentyl-agarose. After PEG precipitation and hydroxyapatite chromatography, the pooled activity (fractions 18–30; Fig. 1a) was precipitated between 40 and 80% ammonium sulphate. The precipitate was dissolved in a known volume (usually 20 ml) of 20 mM-imidazole/HCl (pH 7.1) containing 0.1 mM-DTT and 0.1 mM-EDTA, and the concentration of ammonium sulphate was adjusted to 45% (w/v) by the addition of the solid salt. The turbid suspension was applied to a column of pentyl-agarose equilibrated in the same buffer but containing 45% ammonium sulphate [17]. The column was then washed with buffer containing 30% ammonium sulphate until the absorbance returned to near baseline ($A_{280} < 0.2$). The activity was eluted with a decreasing gradient of ammonium sulphate to 0% and a simultaneously increasing gradient of glycerol to 30%. As shown in Fig. 2, almost all of the Ox-Hb-degrading activity bound to the column and was eluted in one peak.

Our related studies suggested that the Ox-Hb-degrading activity might also hydrolyse insulin (J. M. Fagan & L. Waxman, unpublished work). We therefore assayed fractions from the pentyl-agarose column for insulin-degrading activity. Fig. 2 shows that a major peak of insulin-hydrolysing activity was co-eluted with the Ox-Hb-degrading activity.

F.p.l.c./DEAE. The subsequent purification steps utilized on

Fig. 3. Fractionation of insulin- and Ox-Hb-degrading activities on f.p.l.c./DEAE

Following chromatography on pentyl-agarose, the active fractions were concentrated, dialysed and applied in six separate injections to a GlasPac TSK DEAE-5PW column (8 mm $\times$ 75 mm). Bound proteins were eluted with a linear gradient of NaCl (0–0.5 M). Fractions (1 ml) were collected, and each of the six runs was assayed for insulin-hydrolysing ($\square$) and Ox-Hb-degrading ($\blacksquare$) activity (see Table 1, Fig. 1) and for the hydrolysis of Z-Ala-Arg-Arg-AMC ($\blacktriangle$, as in Fig. 1).

f.p.l.c. system to achieve high-resolution fractionation of the Ox-Hb-degrading activity. Active fractions from the pentyl-agarose step (fractions 75–100) were precipitated with ammonium sulphate (80%, w/v) and the redissolved proteins were dialysed to lower the ionic strength. This material was then applied to a GlasPac TSK DEAE-5PW column equilibrated in 20 mM-Tris/HCl (pH 7.4) and 0.1 mM-DTT. The proteins were then eluted with a gradient of NaCl to 0.5 M. Fig. 3 shows two major peaks of Ox-Hb-degrading activity. One peak (fractions 21–24) was co-eluted with the insulin-degrading activity early in the salt gradient, whereas the second, smaller, peak (fractions 29–34) of Ox-Hb-degrading activity was eluted at a higher salt concentration and did not hydrolyse insulin (Fig. 3a). Both peaks also had associated with them enzymes that hydrolysed Z-Ala-Arg-Arg-AMC (Fig. 3b), a substrate for trypsin-like enzymes and cathepsin B. Fractions 21–24 and 29–34 were pooled separately for further purification.

Chromatography on Mono P. The DEAE fractions containing both the Ox-Hb- and insulin-degrading activities (fractions 21–24) were dialysed against 20 mM-BisTris (pH 6.0) and 0.1 mM-DTT to remove the salt. This material was then applied to a Mono P column which had been used previously to purify the insulin-degrading enzyme [18]. When the column was eluted with a gradient of pH from 5.3 to 4.0, both Ox-Hb- and insulin-degrading activities were found to co-elute between pH 4.5 and pH 5.0 (Fig. 4a). It is also evident that, at this stage of the purification, this enzyme is a small fraction of the total protein,

Fig. 4. Fractionation of Ox-Hb- and insulin-degrading activity by isoelectric point and size

(a) F.p.l.c./DEAE fractions 21–24 that hydrolysed all three substrates tested (Ox-Hb, insulin and Z-Ala-Arg-Arg-AMC) were dialysed and applied in four separate injections to a Mono P column (0.5 cm $\times$ 20 cm) equilibrated in 20 mM-Piperazine/HCl (pH 5.6)/0.1 mM-DTT. Bound proteins were eluted with a pH gradient generated by the application of a 10% solution of Polybuffer 74 containing 0.1 mM-DTT, adjusted to pH 4.2 with HCl. On each of the four runs, fractions (0.5 ml) were collected and assayed for insulin-hydrolysing ($\square$) and Ox-Hb-degrading ($\blacksquare$) activity. pH ($\times$) was monitored at 4 $^{\circ}$ C with a flow-through pH electrode. (b) The Mono P fractions which hydrolysed Ox-Hb and insulin were pooled and the protein was precipitated with 0–80% ammonium sulphate. After resuspending the pellet in 0.45 ml of gel-filtration buffer, 0.2 ml was applied in two runs to two 25 ml columns of Superose 6 connected in tandem and equilibrated in 25 mM-Tris/HCl (pH 7.4), 0.1 mM-DTT, 0.15 M-NaCl and 0.1 mM-EDTA. Fractions (0.5 ml) were collected and assayed for insulin ($\square$) and Ox-Hb ($\blacksquare$) degradation. Inset: the approximate molecular mass (MM) of the Ox-Hb- and insulin-degrading enzyme from erythrocytes was determined using elution volumes of proteins of known molecular mass. The proteins used to calibrate the column were thyroglobulin dimer (1340 kDa) and thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), pyruvate kinase (230 kDa) and lactate dehydrogenase (140 kDa).

since the activity does not correspond to a peak of absorbance at 280 nm (Fig. 4a).

Gel-filtration chromatography. After the Mono P step, the peak of Ox-Hb-degrading activity was concentrated by ammonium sulphate precipitation and fractionated by gel filtration on two columns of Superose 6 connected in tandem. Fig. 4(b) shows that the Ox-Hb- and insulin-degrading activities cannot be separated on the basis of size. When compared with the elution positions of proteins of known molecular mass, the Ox-Hb- and insulin-degrading activities have a mass of 387 kDa (Fig. 4b, inset).

Hydrophobic interaction chromatography on alkyl-Superose. Ammonium sulphate was added to the active fractions from the gel-filtration step to a concentration of 50% (w/v). This material

Fig. 5. Purification of Ox-Hb- and insulin-hydrolysing activity by hydrophobic interaction chromatography on alkyl-Superose

Ammonium sulphate (50%, w/v) was added to pooled Superose 6 fractions that hydrolysed both insulin and Ox-Hb. This material was applied to an alkyl-Superose column (5 mm $\times$ 50 mm) equilibrated in 50 mM-KH₂PO₄ (pH 7)/0.1 mM-DTT/50% ammonium sulphate. Proteins were eluted with a decreasing gradient of ammonium sulphate (50–0%) and collected in 0.5 ml fractions. Fractions were assayed for their ability to hydrolyse Ox-Hb (■), glucagon (□) and Z-Ala-Arg-Arg-AMC (▲).

Fig. 6. SDS/PAGE of three enzymes purified from erythrocytes that hydrolyse peptides, polypeptides and large proteins

Fraction 63 (1 μ g, lane A2; 2.5 μ g, lane A3) and fraction 59 (3 μ g, lane B2) from the alkyl-Superose step (Fig. 5) were electrophoresed on a 10% polyacrylamide gel in the presence of SDS and the proteins were detected with Coomassie Blue. When the gels were restained with silver, no additional bands appeared. The peak fraction from the gel filtration step of the second Ox-Hb-degrading activity (0.2 μ g, lane C2) and 1 μ g of high-molecular-mass multicatalytic proteinase purified from rabbit erythrocytes (lane C1) were electrophoresed on a 12.5% polyacrylamide gel in the presence of SDS. Lanes A1, B1 and C3 show proteins of known molecular mass. Proteins were detected with Coomassie Blue, except for lanes C1–C3, which were stained with silver.

was then applied to a column of alkyl (neopentyl)-Superose, and the proteins were eluted with a gradient of ammonium sulphate from 50% to 0%. This step resolved the activity that hydrolyses Z-Ala-Arg-Arg-AMC, which was eluted at 22% ammonium sulphate, from the Ox-Hb-, glucagon- and insulin-degrading activities (Fig. 5; and results not shown). However, the glucagon- and insulin-degrading activity, which was eluted at 19–20% ammonium sulphate, still co-migrated with the Ox-Hb-degrading protease.

A sample from each peak of activity was electrophoresed on a polyacrylamide slab gel in the presence of SDS. Fig. 6, lane B2, shows that the peak that hydrolyses Z-Ala-Arg-Arg-AMC was purified to a single band. When compared with standards of known molecular mass (lane B1), it had a subunit size of 48–50 kDa. We have been unable to find previous evidence for this enzyme in the literature, and its characterization is under way (J. M. Fagan & L. Waxman, unpublished work). Fig. 6,

Table 2. Effect of inhibitors on the hydrolysis of ¹²⁵I-insulin and Ox-[³H]Hb by the protease isolated from red blood cells

Each inhibitor was preincubated with the enzyme (2.25 μ g) at the concentration indicated for 1 h at room temperature in a volume of 160 μ l containing 50 mM-Tris/HCl (pH 8.0) and DTT (final concentration 2.5 mM). Substrate (3 μ g of ¹²⁵I-insulin or 60 μ g of Ox-[³H]Hb) was added to a final volume of 200 μ l and the mixture was incubated for 1 h at 37 °C. In the case of PCMBs, DTT was not added, since reducing agents reverse the covalent attachment of this reagent to protein thiol groups. Since the stock solutions of some inhibitors were dissolved in Me₂SO, controls were also run which contained this solvent (final concentration 5%, v/v). No preincubation was carried out when the proteins TGF- α , insulin, glucagon and Ox-Hb were tested as competitive substrates, since these might be degraded prior to addition of the radiolabelled substrate. Acid-soluble radioactivity was determined and compared with that in controls treated similarly.

Inhibitor	Inhibition (%)	
	Insulin hydrolysis	Ox-Hb degradation
TGF- α (100 μ M)	65	75
Insulin (0.5 mM)	91	97
Glucagon (0.5 mM)	28	44
Ox-Hb (0.5 mg/ml)	60	50
PCMBs (2 mM)	100	86
EDTA (5 mM)	77	55
Dipyridyl (5 mM)	–11	0
Dipicolinic acid (5 mM)	94	90
Bacitracin (1 mg/ml)	31	50
NEM (2 mM)	96	92
Pepstatin (100 μ M)	10	3
PMSF (1 mM)	15	19
DCIC (100 μ M)	15	9
Chymostatin (100 μ M)	12	6
Ep-475 (100 μ M)	4	10
Leupeptin (100 μ M)	0	7
E-64 (100 μ M)	0	0

lanes A2 and A3, show that the peak of activity that degrades both Ox-Hb and insulin was also purified to homogeneity on alkyl-Superose. It had a subunit molecular mass of 105–110 kDa, and therefore appears to be identical in size to the insulin-degrading activity purified by others from red blood cells [17,18] and *Drosophila* [28].

Inhibitors of the insulin- and Ox-Hb-degrading activity

We tested whether the compounds that inhibited Ox-Hb breakdown in red blood cell extracts ([11]; J. M. Fagan & L. Waxman, unpublished work) also inhibited the activity purified in this study. Table 2 shows that the thiol reagents *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzenesulphonate (PCMBs), the metal chelator EDTA and insulin all inhibited Ox-Hb hydrolysis by the purified enzyme as they did in red blood cell lysates ([11]; J. M. Fagan & L. Waxman, unpublished work). Compounds that had little effect in extracts also failed to inhibit the purified protease, including the aspartic protease inhibitor pepstatin, the serine protease inhibitors phenylmethanesulphonyl fluoride (PMSF), DCIC [29] and chymostatin, and the thiol protease inhibitors E-64, Ep-475 and leupeptin.

We also investigated whether the same compounds that inhibited Ox-Hb hydrolysis by the purified protease also blocked insulin degradation. Insulin and Ox-Hb (non-radioactive), added in large excess over the labelled substrate, and NEM, PCMBs and the metal chelators EDTA and dipicolinic acid, inhibited both Ox-Hb- and insulin-degrading activities. Glucagon, a poly-

Fig. 7. K_m and V_{max} of Ox-Hb degradation by the purified protease

The hydrolysis of Ox-Hb by the purified protease (4.5 μ g) was measured at 37 °C for 2 h. The concentration of Ox-Hb was varied from 0.1 to 2.0 mg in a 200 μ l assay. The K_m was determined from a double-reciprocal plot of the data, and was 2.5 mg/ml. The V_{max} was 145 μ g/h.

peptide which is a substrate for the insulin-degrading enzyme [17,18], was found to inhibit the hydrolysis of both insulin and Ox-Hb, as did bacitracin, an inhibitor of the insulin-degrading enzyme [18]. Transforming growth factor- α (TGF- α), a polypeptide shown to have a very high affinity for the analogous insulin-degrading enzyme from *Drosophila* cells [30], also inhibited the breakdown of both insulin and Ox-Hb. Those compounds which had little effect on the hydrolysis of insulin, including the chelator dipyridyl, and several inhibitors of serine and thiol proteases, also inhibited Ox-Hb degradation poorly.

K_m for Ox-Hb hydrolysis

Further evidence that the enzyme purified in this investigation is responsible for the breakdown of Ox-Hb in lysates was obtained by comparing the K_m for Ox-Hb hydrolysis in lysates with that for the purified enzyme. As shown in Fig. 7, the apparent K_m for the purified enzyme was 2.5 mg/ml, with a V_{max} of 145 μ g/h. In contrast, the apparent K_m values for small polypeptide substrates are much lower. The K_m which has been reported for insulin is 100 nM (0.65 μ g/ml) [17,18], and for glucagon the K_m is 1.5–2.0 μ M (5–7 μ g/ml) [18]; results not shown). However, it may not be valid to compare the apparent K_m values for large protein substrates with those for small peptide substrates, since more cleavages are probably required to solubilize a molecule of Ox-Hb than a molecule of insulin.

Other protein substrates

We determined whether the protease that degrades Ox-Hb is also able to hydrolyse other protein substrates to acid-soluble fragments. Table 3 shows the rate of breakdown of several radiolabelled protein substrates determined at two concentrations. The low-molecular-mass polypeptides insulin and glucagon are not degraded any more rapidly at 24 μ g/assay (120 μ g/ml) than at 6 μ g/assay (30 μ g/ml), which is consistent with the low K_m values for these proteins as substrates. By comparison, the release of acid-soluble fragments from larger protein substrates, including lysozyme (14.3 kDa), Ox-Hb (66 kDa), casein (24 kDa), globin (16 kDa) and denatured BSA (66 kDa), was slow (less than 0.2 μ g/h) when these proteins were added at 6 μ g/assay. However, when the amount of these substrates was increased to 24 μ g/assay, the activity of the protease towards them increased 5–8-fold. It therefore appears that this enzyme will hydrolyse both small polypeptides and large proteins.

Table 3. Degradation of various polypeptides and proteins by the protease isolated from red blood cells

Purified enzyme (2.25 μ g) was incubated with each protein substrate (6 or 24 μ g) in a reaction volume of 200 μ l which contained 50 mM-Tris/HCl (pH 8.0), 5 mM-MgCl₂ and 0.1 mM-DTT. After 1 h at 37 °C, proteolysis was terminated by the addition of 25 μ l of 10% BSA and 575 μ l of 10% trichloroacetic acid (22% trichloroacetic acid when glucagon and insulin breakdown was measured). After centrifugation, a sample of the supernatant was counted for radioactivity in a liquid scintillation or γ -radiation spectrometer.

Substrate	Substrate added to the assay (μ g)...	Proteolysis (μ g/h)	
		6	24
¹²⁵ I-Insulin		1.37	1.48
[³ H]Lysozyme		0.16	1.36
Ox-[³ H]Hb		0.16	0.61
[³ H]Casein		0.07	0.39
[³ H]Globin		0.02	0.12
¹²⁵ I-Glucagon		4.08	3.64
Denatured [³ H]BSA		0.17	1.00

Further characterization of the second Ox-Hb-degrading activity

The second peak of Ox-Hb-degrading activity (fractions 29–34) was pooled from the TSK-DEAE step (Fig. 3) and concentrated with 80% (w/v) ammonium sulphate. When this material was applied to gel filtration on Superose 6, the Ox-Hb-degrading activity co-migrated with the hydrolysis of a variety of peptide fluorogenic substrates, including Z-Ala-Arg-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-MNA ([15,27]; results not shown). Its molecular mass on this column was ~ 700 kDa, which is the same size as the multicatalytic proteinase purified from red blood cells (results not shown). However, in contrast with the amount of multicatalytic proteinase obtained from 1 litre of packed red blood cells (> 5 mg), it was possible to obtain only 5 μ g of this new activity which hydrolysed peptides and degraded Ox-Hb.

The active fractions from the Superose 6 column were pooled and analysed by SDS/PAGE (Fig. 6, lane C2). When stained with silver, this material showed a cluster of bands ranging in size from 22 to 30 kDa. When the multicatalytic proteinase purified from the same lysate was run on a gel and stained with silver, a similar pattern of bands was obtained, but with slightly different molecular masses (Fig. 6, lane C1). It is likely that the high-molecular-mass proteinase isolated in this study is related to the multicatalytic proteinase [15].

DISCUSSION

The only well-characterized proteolytic pathway in reticulocytes is the ATP- and ubiquitin-dependent degradative pathway. This degradative system is thought to be important in the breakdown of abnormal proteins and proteins lost during maturation [1]. Red blood cells rapidly degrade proteins exposed to a variety of oxidizing agents [4,5,7]. However, the breakdown of oxidant-damaged proteins in red blood cells does not require ATP or ubiquitin [5]. Therefore red blood cells must obtain a second major degradative system, but one that does not require metabolic energy.

Efforts to identify the proteases that participate in the breakdown of oxidatively damaged proteins have been limited. Several proteases have been purified from red blood cells and characterized in some detail, although their physiological roles

remain unknown. By the use of inhibitors of the various classes of proteases, we and others have determined some of the properties of an ATP-independent proteolytic pathway [5,7,11,13]. We systematically examined the role that each of the known red blood cell proteases might play in degrading Ox-Hb and were able to eliminate as possible candidates the calpains, the multicatalytic proteinase, aspartic proteases and most thiol and serine proteases [11]. However, metal chelating agents and insulin inhibited the breakdown of Ox-Hb in red blood cell extracts [11], suggesting that a metal-dependent insulinase may play a key role in this process.

The insulin-degrading activity purified by others [17,18,28] appears to be identical to the Ox-Hb-degrading activity purified here. It has a high affinity for insulin and glucagon, it is inhibited by metal chelating agents and thiol reagents, and it has a subunit size of 105–110 kDa. Like the human enzyme [31], the Ox-Hb-degrading activity from rabbit erythrocytes had a blocked *N*-terminus (L. Waxman & J. M. Fagan, unpublished work). The insulin-degrading activity has been reported to be either a monomer, based on sedimentation in a sucrose density gradient [28], or a dimer or trimer on gel filtration [17,18]. Using a variety of different gel filtration media and by gel electrophoresis under non-denaturing conditions, we have also obtained a range of values for the molecular mass, corresponding to a dimer, trimer or tetramer (L. Waxman & J. M. Fagan, unpublished work). One possible explanation for this discrepancy is that the enzyme may exist in a rapid equilibrium between several states of aggregation, all of which are active.

Degradation of Ox-Hb by the purified enzyme is similar in many respects to Ox-Hb breakdown in crude erythrocyte extracts. Compounds that inhibit Ox-Hb and insulin degradation by the purified protease also blocked Ox-Hb breakdown in lysates. Compounds that had no effect on the activity of the enzyme had no effect in lysates. In addition, the K_m for Ox-Hb as substrate was similar for lysates and for the purified enzyme.

Until this report there were two views on the physiological role of the insulin-degrading enzyme. The first was based on experiments which indicated that the enzyme efficiently hydrolysed small polypeptides but not large proteins [17]. According to this model, (a) intracellular proteases attack cell proteins to generate fragments, which are (b) cleaved to small peptides by this enzyme, which are then (c) hydrolysed to amino acids by peptidases. The second view, based on the fact that its K_m for small polypeptide substrates including insulin and glucagon is 1 μ M or less, is that the protease functions intracellularly to inactivate hormones such as insulin [32]. Several experiments have provided strong evidence for this proposal, including the following: (1) microinjection of monoclonal antibodies against the protease inhibits the degradation of intracellular insulin [32]; (2) the cleavage sites of insulin obtained with the purified protease are identical with those observed in insulin extracted from cells [33,34]; (3) insulin can be cross-linked in intact cells to the protease [35]; and (4) receptor-bound insulin is a substrate for the protease [36].

Based on the observations presented here, we propose that this enzyme may also serve a very different function. We suggest that it is a key component of a high- K_m intracellular cytoplasmic degradative pathway. Such a system would be required when abnormal proteins are generated in large amounts, such as might occur when red blood cells are exposed to oxidants. In contrast, other degradative pathways with more specialized functions might have low K_m values for substrates. One of these may be the ATP+ubiquitin-dependent degradative pathway which is proposed to degrade short-lived proteins and certain kinds of abnormal proteins [1]. Although the K_m for a substrate of the ATP-dependent pathway *in vivo* is not available, the K_m for

lysozyme, the protein substrate commonly used to study this pathway *in vitro* in reticulocyte lysate, is 30 μ g/ml (2 μ M) (L. Waxman & J. M. Fagan, unpublished work). Whether other intracellular proteases such as the calpains and the multicatalytic proteinase are components of a high- K_m or low- K_m proteolytic pathway is still to be determined.

In this study we have also isolated a high-molecular-mass ($\sim$ 700 kDa) protease which degrades Ox-Hb as well as a variety of fluorogenic peptide substrates. It resembles the multicatalytic proteinase purified from many mammalian tissues in its size, its activity towards a variety of trypsin-like, chymotrypsin-like and other fluorogenic peptide substrates, and its sensitivity towards certain inhibitors (L. Waxman & J. M. Fagan, unpublished work). However, the high- M_r protease purified here binds weakly to hydroxyapatite and was well resolved from the multicatalytic proteinase that binds tightly to this chromatographic medium. The subunit structure of the high- M_r protease isolated in this study and the one purified by standard procedures [15] also show striking similarities on SDS/PAGE, although the two do not appear to be identical. Possibly this peptide- and Ox-Hb-degrading activity represents a minor form of the high- M_r multicatalytic proteinase isolated previously [15] which chromatographs differently due to a post-synthetic modification. The role of this enzyme in the breakdown of Ox-Hb and its relationship to the multicatalytic proteinase is presently unclear. However, immunoprecipitation of the multicatalytic proteinase did not affect the breakdown of Ox-Hb in erythrocyte lysates (J. M. Fagan & L. Waxman, unpublished work), suggesting that the multicatalytic proteinase plays little or no role in the hydrolysis of Ox-Hb in erythroid cells.

Whether the enzymes that degrade oxidatively damaged proteins in red blood cells play a similar role in other mammalian cells is not known. Possibly this proteolytic system is unique to red blood cells because they contain one predominant, readily oxidizable, protein and because red blood cells lack lysosomes. However, insulin-degrading enzyme with a subunit of 110 kDa has been found in a variety of mammalian cells in addition to red blood cells. Therefore it may serve a similar function in other cells. Since the cDNA for this enzyme has been isolated [31], it may be possible to test by genetic means whether this enzyme plays a role in removing proteins damaged by oxidants and free radicals in other tissues. Alternatively, highly selective inhibitors can be designed when more becomes known about the specificity of this protease and utilized to test its role *in vivo*.

We are grateful to Ms. Carole Hannan and Ms. Dorothy Bonenberger for their assistance in preparing this manuscript. This work has been supported by research grants from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, the United States Department of Agriculture, the American Heart Association and the New Jersey Agricultural Experiment Station, which is supported by State funds. This work was done during the tenure of an Established Investigatorship by J. M. F. from the American Heart Association.

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Received 27 February 1991; accepted 19 March 1991