Proteolysis of *N*-ethylmaleimide-modified aldolase loaded into erythrocyte ghosts: prevention by inhibitors of calpain

Melvyn F. HOPGOOD*, Spencer E. KNOWLES and F. John BALLARD
CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide, SA 5000, Australia

1. When rabbit muscle aldolase labelled with tritium and inactivated by *N*-ethylmaleimide (NEM) was loaded into erythrocyte ghosts, significant proteolysis of the loaded protein occurred. The major product of this proteolysis, separated by electrophoresis under dissociating conditions, was found to be approx. 2 kDa smaller than the parent protein. 2. Proteolysis was detectable during erythrocyte ghost loading at 0 °C, reaching a plateau after approx. 12 min. Subsequent incubation at 37 °C to allow resealing of the ghosts resulted in additional proteolysis, and up to 20 % of the loaded protein was converted to the smaller 38 kDa derivative. 3. EDTA, EGTA, leupeptin and chymostatin, each inhibitors of calcium-activated neutral proteinases (calpains), were the most effective inhibitors of the proteolysis of NEM-inactivated aldolase in ghosts. Other proteinase inhibitors were ineffective, while phenylmethanesulphonyl fluoride was only partially effective. 4. Inhibition of the proteolysis by EGTA was prevented by CaCl₂, supporting the involvement of erythrocyte calpain. 5. Pretreatment of ghosts with EGTA prior to loading of NEM-modified aldolase followed by microinjection of the protein into HeLa cells did not result in a different rate of its overall breakdown to acid-soluble products. EGTA is suggested as a useful agent for the erythrocyte ghost-mediated microinjection of calpain-sensitive proteins.

INTRODUCTION

Erythrocytes contain a variety of proteinases and proteolytic systems, both soluble (Goldberg & Boches, 1982; Melloni *et al.*, 1982b; Fagan *et al.*, 1986) and membrane-associated (Tökés & Chambers, 1975; Allen & Cadman, 1979; Murakami *et al.*, 1979; Siegel *et al.*, 1980) which may be involved in protein turnover or processing. It is not surprising, therefore, that some proteins are found to be hydrolysed when loaded into erythrocyte ghosts (Neff *et al.*, 1981; Rechsteiner, 1982; Backer *et al.*, 1983; McElligott & Dice, 1984; Netland & Dice, 1985; Hopgood *et al.*, 1988) prior to erythrocyte ghost-mediated microinjection.

We have been using erythrocyte ghosts as a vehicle to microinject ³H-labelled aldolase and sulphhydryl-modified forms of the enzyme into HeLa cells, as part of a series of investigations into the mechanism of breakdown of normal intracellular proteins. Sulphydryl-modified aldolase, particularly that modified with *N*-ethylmaleimide (NEM), was hydrolysed when loaded into erythrocyte ghosts (Hopgood *et al.*, 1988). This hydrolysis resulted in the microinjection of a mixture of proteins rather than a single species. We now report investigations of the nature of the proteolytic reactions involved and their inhibition by adding proteinase inhibitors to the erythrocyte ghosts before loading of the protein. We have also used inhibitor-loaded ghosts as a vehicle for microinjection, and have measured the rate of breakdown of proteins injected into HeLa cells in the presence and absence of a selected inhibitor, EGTA.

MATERIALS AND METHODS

Materials

Phenylmethanesulphonyl fluoride (PMSF), 1-chloro-4-phenyl-3-tosylamidobutan-2-one (TPCK), 1-chloro-7-amino-3-tosylamidoheptan-2-one (TLCK) and EGTA were from Sigma Chemical Co., St. Louis, MO, U.S.A. Leupeptin, pepstatin and bestatin were from Peptide Institute Inc., Osaka, Japan. Phosphoramidon and chymostatin were kindly provided by Dr. H. Umezawa, Microbial Chemistry Research Foundation, Tokyo, Japan. The sources of all other materials were as previously described (Knowles *et al.*, 1988; Hopgood *et al.*, 1988).

Labelling and modification of aldolase

Rabbit muscle aldolase (fructose-bisphosphate aldolase; EC 4.1.2.13) was labelled with ³H by reductive methylation and modified by treatment with NEM as previously described (Hopgood *et al.*, 1988). All batches of NEM-modified aldolase (NEM-aldolase) used in this study retained between 1 % and 5 % of original catalytic activity.

Loading of proteins into erythrocyte ghosts

Aldolase or NEM-aldolase were loaded into human erythrocyte ghosts as described previously (Knowles *et al.*, 1988), except that in some cases the preswollen ghosts were preincubated on ice for 5 min with a proteinase inhibitor before addition of the hypo-osmotic protein solution. The concentration given for each inhibitor was

Abbreviations used: NEM, *N*-ethylmaleimide; NEM-aldolase, NEM-modified (inactivated) aldolase; PMSF, phenylmethanesulphonyl fluoride; TPCK, 1-chloro-4-phenyl-3-tosylamidobutan-2-one; TLCK, 1-chloro-7-amino-3-tosylamidoheptan-2-one.

* To whom correspondence should be addressed.

that present during the loading phase (2 min at 0 °C) and during resealing (60 min at 37 °C). The resealed ghosts were then washed with phosphate-buffered saline (Rechsteiner, 1982) to which inhibitors were added where indicated at the same concentration used during loading and resealing.

Analysis of proteolysis by erythrocyte ghosts

Samples of labelled proteins and loaded ghosts were taken at various stages of loading and immediately frozen. They were thawed in SDS/dithiothreitol (Chandler & Ballard, 1985) and incubated immediately at 95 °C for 5 min. The dissociated polypeptide mixtures were separated by polyacrylamide-gel electrophoresis as described earlier (Chandler & Ballard, 1985), either on 10–25% acrylamide gradients or on linear 10% gels. After separation, radioactive peptides were detected by fluorography (Chandler & Ballard, 1985). Molecular masses of separated peptides were estimated by reference to the mobilities of ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) standards. Areas of the gels corresponding to 38–41 kDa, 36–38 kDa, 33–36 kDa and 30–33 kDa were excised such that the major fluorographic bands were included in each area and the junction between areas was a region of minimal fluorographic intensity. Radioactivity in the 36–38 kDa area (hereafter called the 38 kDa band) of the starting protein was expressed as a percentage of that in the 38–41 kDa area (40 kDa monomer band), and proteolysis was assessed from the increase in this ratio.

Fusion of loaded ghosts to HeLa cells

Loaded ghosts were fused to HeLa cells with the aid of inactivated Sendai virus, washed and plated out as described previously (Knowles *et al.*, 1988). Following a 6 h incubation to allow reattachment as monolayer cultures, the residual ghosts were lysed and removed (Knowles *et al.*, 1988).

Breakdown of injected proteins

Overall breakdown of injected proteins to trichloroacetic acid-soluble products between 6 h and 42 h after microinjection into HeLa cells was measured and expressed as described previously (Knowles *et al.*, 1988).

RESULTS

Labelled NEM-aldolase contained a small proportion of contaminating peptides (approx. 10%) which separated from the 40 kDa monomer during electrophoresis under dissociating conditions (Fig. 1, lane 1). These contaminants persisted through the erythrocyte loading procedure and were evident at approximately the same proportion in all samples. In contrast, radioactive peptides whose mobility indicated a size reduction of about 2 kDa were generated after only 30 s exposure (Fig. 1, lane 5) of the protein to the ghosts during swelling and protein loading. The proportion of material at about 38 kDa increased somewhat during prolonged incubations at 0 °C, but was no greater after 30 min than after 12 min (Fig. 1, compare lanes 2 and 3). Incubation at 37 °C for 60 min to allow complete resealing of the loaded ghosts resulted in a further increase in the proportion of material at 38 kDa (Fig. 1, lane 7). The final preparation of ghosts, washed free of

Fig. 1. Proteolysis of NEM-modified aldolase during loading into erythrocyte ghosts

³H-labelled aldolase was prepared and inactivated by reaction with NEM as described in the Materials and methods section. NEM-aldolase was incubated with preswollen erythrocyte ghosts at 0 °C for between 30 s and 30 min. Ghosts incubated for 2 min were resealed by restoration of iso-osmolarity and incubated for 1 h at 37 °C, and the resealed ghosts were washed with phosphate-buffered saline with three centrifugation steps. Samples at each stage were dissolved and dissociated in SDS/dithiothreitol, separated by polyacrylamide-gel electrophoresis (10% acrylamide); and the radioactive peptides were visualized by fluorography, excised from the gels and quantified by liquid scintillation counting. Values for radioactivity in the 38 kDa band, expressed as a percentage of the 40 kDa band, are shown in parentheses.

protein which had not been loaded, contained the 38 kDa material as the only significant contaminant of size above 14 kDa (Fig. 1, lane 6).

Various proteinase inhibitors were added to the ghosts before loading NEM-aldolase, in an attempt to prevent generation of the 38 kDa material (Fig. 2*a,b*). Leupeptin, EDTA [Fig. 2*a*], lanes 3 and 5 respectively], EGTA and chymostatin [Fig. 2*b*], lanes 2 and 4 respectively] were particularly effective inhibitors of the proteolytic cleavage. PMSF was less effective [Fig. 2*a*], lane 7; Fig. 2*b*], lane 3]. Pepstatin and phosphoramidon [Fig. 2*a*], lanes 4 and 6 respectively] were ineffective, as were TPCK, TLCK and bestatin (results not shown). The degree of inhibition by each agent was quantified by measuring radioactivity separated in the 38 kDa and 40 kDa bands of the original protein and in ghosts loaded in the presence and absence of the inhibitor (Table 1). In this particular preparation of NEM-aldolase, radioactivity separated at 38 kDa was approximately 4% of that at 40 kDa. This proportion increased nearly 5-fold to 19% during the loading of that protein into ghosts, but increased to only 7.3% in the presence of the most effective inhibitors (EDTA and

Fig. 2. Effect of proteinase inhibitors on proteolysis of NEM-aldolase by erythrocyte ghosts

Preswollen erythrocyte ghosts were incubated with and without inhibitors for 5 min prior to addition of NEM-aldolase. Final concentrations of inhibitors were: leupeptin, pepstatin and phosphoramidon, 0.2 mg/ml; EDTA and EGTA, 5.0 mM; PMSF, 2 mM and chymostatin 0.06 mg/ml. After loading, resealing and washing, samples of ghosts were subjected to polyacrylamide-gel electrophoresis and separated peptides detected by fluorography. (a) Gradient gel: 10–25 % acrylamide; (b) linear gel: 10 % acrylamide.

Fig. 3. Effect of concentration of EGTA on proteolysis of NEM-aldolase by erythrocyte ghosts

Ghosts were preincubated with various concentrations of EGTA for 5 min prior to loading NEM-aldolase. Proteolysis which occurred during loading and resealing was assessed after polyacrylamide-gel electrophoresis (10% acrylamide) by excising areas of the gel and measuring the radioactivity therein. Inhibition of proteolysis was calculated as described in the Materials and methods section.

Table 1. Inhibition of generation of 38 kDa material from NEM-aldolase in erythrocyte ghosts

Muscle aldolase was labelled with ^3H , modified with NEM and loaded into erythrocyte ghosts as described in the Materials and methods section. Proteinase inhibitors, where used, were added to the preswollen ghosts 5 min before addition of the protein. Samples were separated by polyacrylamide-gel electrophoresis and areas of the gels corresponding to molecular weight ranges 36–38 kDa (38 kDa band) and 38–41 kDa (40 kDa band) were excised and their radioactivities compared. Values are means $\pm$ S.E.M. for the number of experiments given in parentheses.

Additions at loading	Radioactivity in 38 kDa band (% of 40 kDa)	Inhibition of proteolysis (%)
None	19.0 $\pm$ 4.2* (5)	–
PMSF (2 mM)	13.9 $\pm$ 3.6 (4)	35 $\pm$ 9 (4)
EGTA (5 mM)	7.2 $\pm$ 1.5 (5)	81 $\pm$ 4 (5)
EDTA (5 mM)	7.3 $\pm$ 2.1 (6)	80 $\pm$ 5 (6)
Leupeptin (0.5 mg/ml)	8.6 $\pm$ 1.6 (6)	71 $\pm$ 4 (6)
Leupeptin + EDTA	7.3 $\pm$ 1.8 (4)	80 $\pm$ 5 (4)

* Before loading, 4.4 $\pm$ 0.7 (5).

EGTA) and to only 8.6% with leupeptin (Table 1). The addition of EDTA and leupeptin together did not increase the inhibition (Table 1). The inhibition was not

significantly increased by raising the concentration of the inhibitors or by adding them to the washing solutions after resealing (results not shown). Quantification of the degree of proteolysis also showed that PMSF was a partial inhibitor (Table 1), though its effect was less than one-half that of the metal chelators EDTA and EGTA.

EGTA was an effective inhibitor of the generation of 38 kDa material at a range of concentrations from 0.5 to 5.0 mM (Fig. 3). Inhibition at 0.5 mM-EGTA was 70% of that observed at 5.0 mM. In other experiments (see Fig. 5; other results not shown) EGTA at 1.0 mM inhibited the limited proteolysis of NEM-aldolase by 70–80%, equivalent to the effect observed here with 5.0 mM-EGTA. The possible involvement of calcium ions in the proteolysis was examined by adding CaCl_2 in the presence of sufficient EGTA (2.5 mM) to cause maximal inhibition of the reaction. Addition of 1.25 mM- CaCl_2 (Fig. 4, lanes 2 and 6) only slightly relieved the inhibition. When equimolar amounts of EGTA and CaCl_2 were present (2.5 mM, lanes 3 and 7), extensive proteolysis was observed. Proteolysis was increased even further when 5.0 mM- CaCl_2 was included. This result was clear with the sample of resealed ghosts (Fig. 4, lane 4) but was disguised in the case of washed ghosts (lane 8) because a smaller amount of radioactivity was applied to this lane. Excising and counting of the bands of lane 8 revealed the increase in proportion of 38 kDa material (results not shown).

A sample of ^3H -labelled aldolase and its NEM-

Fig. 4. Reversal of EGTA inhibition by calcium ions

Ghosts were preincubated with 2.5 mM-EGTA plus 0, 1.25, 2.5 or 5.0 mM- CaCl_2 for 5 min prior to the addition of NEM-aldolase. Samples of ghosts after 1 h resealing and after resealing plus washing were subjected to polyacrylamide-gel electrophoresis (10% acrylamide) and the radioactive peptides were visualized by fluorography.

Table 2. Rates of degradation to acid-soluble products of aldolase and NEM-modified aldolase after microinjection into HeLa cells

^3H -Labelled aldolase and its NEM-modified form were loaded into erythrocyte ghosts in the presence or absence of 1 mM-EGTA and the ghosts were fused to HeLa cells. Overall degradation of the microinjected proteins to trichloroacetic acid-soluble products was measured and expressed as described in the Materials and methods section. Values are means $\pm$ S.E.M. for three determinations. The washed ghosts analysed in Fig. 5 were used for this experiment.

Incubation time (h)	Percent degradation of:		
	Aldolase + EGTA	NEM-aldolase	
		+ EGTA	- EGTA
9	2.03 $\pm$ 0.08	1.72 $\pm$ 0.09	1.56 $\pm$ 0.15
18	6.58 $\pm$ 0.45	6.13 $\pm$ 0.27	6.32 $\pm$ 0.19
27	9.07 $\pm$ 0.68	9.28 $\pm$ 0.30	8.36 $\pm$ 0.44
36	11.85 $\pm$ 0.38	12.22 $\pm$ 0.60	10.86 $\pm$ 0.05

modified form were each loaded into erythrocyte ghosts in the presence of EGTA (the latter protein also in the absence of EGTA) and the ghosts were fused to HeLa

Fig. 5. Comparison of aldolase and NEM-aldolase loaded into erythrocyte ghosts in the presence and absence of EGTA

Preswollen erythrocyte ghosts were preincubated in the presence and absence of 1 mM-EGTA for 5 min prior to loading aldolase or its NEM-inactivated form. Samples of proteins before and after loading were separated by gradient polyacrylamide-gel electrophoresis (10–25% acrylamide) and peptides were visualized by fluorography.

cells to determine whether the microinjected proteins were degraded at the same or different rates. Electrophoretic analysis of the loaded proteins revealed little or no proteolysis of aldolase (Fig. 5, lanes 1 and 3) but extensive proteolysis of NEM-aldolase (Fig. 5, lanes 2 and 4) which was reduced as expected in the presence of EGTA (Fig. 5, lane 5).

Overall degradation of aldolase and NEM-aldolase to trichloroacetic acid-soluble products occurred at a similar rate (Table 2). Aldolase loaded in the presence of EGTA, and NEM-aldolase loaded in the presence or absence of EGTA, were all degraded after microinjection into HeLa cells, at an average rate of 0.3% per hour (Table 2), measured over the period 6–42 h after microinjection. We also observed a lower overall breakdown rate over the first 9 h period in all three cases.

DISCUSSION

The stability of proteins loaded into erythrocyte ghosts has commonly been assessed either by gel electrophoresis of the loaded protein or by quantification of any acid-soluble products which may be produced. The latter technique is more usual, but indicates only that proteolysis has proceeded to low-molecular-mass products, perhaps free amino acids, whereas the former method can show that a cleavage of the loaded protein has produced large fragments that are not acid-soluble.

For instance, Netland & Dice (1985) showed that ^{125}I -labelled glyceraldehyde 3-phosphate dehydrogenase, after loading into erythrocyte ghosts, was almost entirely converted to smaller peptide fragments, but only 10% was degraded to acid-soluble material. These authors also reported significant degradation of ^3H -labelled aldolase to acid-soluble products (20% during loading and resealing), but did not subject the loaded aldolase to electrophoretic analysis. We have found that several batches of ^3H -labelled aldolase are stable by both criteria (Hopgood *et al.*, 1988). The reason for this discrepancy between our results and those of Netland & Dice (1985) is unclear, though the aldolase sample was from a different supplier.

In contrast to the situation we find with aldolase, there is considerable proteolytic cleavage of NEM-aldolase during loading into erythrocyte ghosts. Only one major product, that of molecular mass of approx. 38 kDa, is detected on electrophoresis (Figs. 1 and 2a), suggestive of a single proteolytic event which removes a small peptide of approx. 2 kDa from the 40 kDa monomer. Additional but quantitatively minor peptides of approx. masses of 35 and 31 kDa were also detected in loaded ghosts (Figs. 2(b) and 5), as in previous experiments (Hopgood *et al.*, 1988), indicating that further proteolysis also occurs. The presence and activity of such minor proteinases could account for the lack of complete inhibition by the chelating agents and leupeptin of the cleavage which produces the 38 kDa material, as well as for at least a part of the inhibition observed with PMSF.

Certain specific proteinase inhibitors have no effect on the generation of 38 kDa material from NEM-aldolase. From these results we conclude that aminopeptidases (inhibitor: bestatin; Umezawa & Aoyagi, 1983), aspartic proteinases (inhibitor: pepstatin; Umezawa & Aoyagi, 1983) and thermolysin-type metalloproteinases (inhibitor: phosphoramidon; Bond & Butler, 1987) are unlikely to be involved. Since serine proteinases are generally inhibited by one of the chloromethyl ketones (Shaw, 1967) or by PMSF (Bond & Butler, 1987), the lack of effect of TPCK and TLCK and the partial inhibition by PMSF suggest that the involvement of serine proteinases is minor.

Several lines of evidence presented here support the conclusion that the proteolysis of NEM-aldolase is catalysed by a calpain. Thus proteolysis was inhibited by leupeptin, by the metal chelators EDTA and EGTA, and by chymostatin (Figs. 2a, 2b), all of which are effective inhibitors of calpain (Melloni *et al.*, 1982a; Pontremoli & Melloni, 1986). Furthermore, the involvement of calcium ions in the reaction is illustrated by the reversal of EGTA inhibition by added CaCl_2 (Fig. 4).

Calpain I activity has been reported in human erythrocytes (Hatanaka *et al.*, 1984). The activity is found almost entirely in the cytosol, but a small proportion (2%) is associated with the erythrocyte membrane, in which site it is not subject to inhibition by calpastatin, its natural inhibitor (Hatanaka *et al.*, 1984). Calpastatin is present in excess over calpain I in the erythrocyte cytosol (Murakami *et al.*, 1981; Hatanaka *et al.*, 1984), though both would be expected to be much reduced in ghosts due to losses of cytosol contents during hypo-osmotic loading. We suggest that NEM-aldolase may be, by reason of its altered structure, much more susceptible than the native enzyme to hydrolysis by calpain. The rapid and limited hydrolysis which occurs

during loading of the modified enzyme into erythrocyte ghosts at 0 °C supports the proposition that exposure of the protein to calpain is very brief – perhaps it is the membrane-associated erythrocyte calpain which catalyses the observed proteolysis. Consequently the NEM-aldolase would be exposed to this calpastatin-insensitive calpain activity predominantly during its passage through or near the erythrocyte membrane whilst loading is occurring. The subsequent increase in proteolysis which occurs during resealing at 37 °C is relatively slower, perhaps due to limited access of cell membrane-associated calpain to the loaded protein in the ghost interior.

If EGTA is transferred during microinjection to the recipient HeLa cell along with the modified aldolase, and perturbs protein breakdown processes therein, then it will be of limited usefulness. We find, however, that EGTA does not modify the rate of breakdown of injected NEM-aldolase to trichloroacetic acid-soluble products (Table 2). The slower breakdown rate observed in the first 9 h period, which is most probably a consequence of the fusion process (Knowles *et al.*, 1989) is also consistently observed in this study. The lack of effect of EGTA may be due to its leakage from the loaded ghosts, dilution in the recipient cell cytoplasm, or simply that it may not be a significant modulator of protein breakdown in the HeLa cell. Regardless of the precise mechanism involved, it is evident that EGTA is a suitable agent for the prevention of proteolysis of calpain-sensitive proteins during loading into erythrocyte ghosts prior to microinjection studies.

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