Biochem. J. (1988) 256, 81–88 (Printed in Great Britain)

Degradation of native and modified forms of fructose-bisphosphate aldolase microinjected into HeLa cells

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The uptake and degradation of radiolabelled rabbit muscle fructose-bisphosphate aldolase (EC 4.1.2.13) was studied in HeLa cells microinjected by the erythrocyte ghost fusion system. Labelled aldolase was progressively modified by treatment with GSSG or *N*-ethylmaleimide (NEM) before microinjection to determine whether these agents, which inactivate and destabilize the enzyme *in vitro*, affect the half-life of the enzyme *in vivo*. Increasing exposure of aldolase to GSSG or NEM before microinjection increased the extent of aldolase transfer into the HeLa cells and decreased the proportion of the protein that could be extracted from the cells after water lysis. Some degradation of the GSSG- and NEM-inactivated aldolases was observed in the ghosts before microinjection; thus a family of radiolabelled proteins was microinjected in these experiments. In spite of the above differences, the 40 kDa subunit of each aldolase form was degraded with a half-life of 30 h in the HeLa cells. In contrast, the progressively modified forms of aldolase were increasingly susceptible to proteolytic action *in vitro* by chymotrypsin or by cathepsin B and in ghosts. These studies indicate that the rate of aldolase degradation in cells is not determined by attack by cellular proteinases that recognize vulnerable protein substrates; the results are most easily explained by a random autophagic process involving the lysosomal system.

INTRODUCTION

Our knowledge about the mechanisms and routes of degradation of intracellular proteins remains limited, in spite of a substantial amount of research in this area (Ballard, 1977; Hershko & Ciechanover, 1982; Khairallah *et al.*, 1985; Mayer & Doherty, 1986; Beynon & Bond, 1986; Glaumann & Ballard, 1987). Several investigators have suggested that the post-translational modifications of proteins are determinative in the initial stages of the breakdown process. Post-translational modifications suggested to be determinative are ubiquitinylation (Ciechanover *et al.*, 1984), oxidation (Levine *et al.*, 1981) and deamidation (Robinson *et al.*, 1970). Oxidative events that have been implicated in initiating extensive degradation include oxidation of essential histidine residues by mixed-function oxidation systems (Levine, 1983), attack by oxygen-derived free radicals (Wolff *et al.*, 1986), and oxidation of cysteine residues by disulphides (Francis & Ballard, 1980; Offerman *et al.*, 1984).

Rabbit muscle fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) has been studied *in vitro* as a model for soluble enzymes with relatively long biological half-lives, to determine what factors may play a role in the stability and proteolytic vulnerability of the protein. The native enzyme is readily inactivated *in vitro* by several proteinases such as cathepsins B, D, L, papain, chymotrypsin or meprin via limited *C*-terminal proteolysis (Offerman *et al.*, 1984; Bond & Offerman, 1981), but the bulk of the protein is relatively resistant to proteolysis. However, reactions of aldolase with disulphides markedly destabilize the protein and increase its susceptibility to complete proteolysis (Offerman *et al.*, 1984). In the present studies we have examined whether progressive inactivation of aldolase by oxidation with GSSG or by modification of cysteine thiol groups with *N*-ethylmaleimide (NEM) increases the rate of degradation of the enzyme *in vivo*. The native and GSSG- or NEM- modified forms of aldolase were microinjected into HeLa cells and their degradation was monitored.

MATERIALS AND METHODS

Materials

Aldolase (rabbit muscle) and chymotrypsin A (bovine pancreas) were obtained from Boehringer Mannheim P/L, North Ryde, N.S.W., Australia. Bovine serum albumin was from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine liver cathepsin B was gift from Dr. Alan J. Barrett (Strangeways Laboratory, Cambridge, U.K.). The sources of other chemicals and materials were as previously described (Knowles *et al.*, 1988).

Labelling of aldolase

Aldolase was labelled with ^3H by reductive methylation (Tack *et al.*, 1980), separated from reaction products on Sephadex G-25M equilibrated with 5 mM-sodium phosphate, pH 7.4, and stored in liquid N_2. The specific radioactivity of the protein was 670 μCi/mg, representing 4.0 [^{3}H]methyl groups added per molecule of aldolase. Spectrophotometric assay (Blostein & Rutter, 1963) showed that the enzyme retained approximately half of its original catalytic activity after labelling.

Abbreviation used: NEM, *N*-ethylmaleimide.

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Modification of labelled aldolase

Thiol groups of labelled aldolase were oxidized with 12 mM-GSSG at 37 °C (Offerman *et al.*, 1984) for up to 3 h, followed by storage on ice for up to 2 h. Reaction with 1 mM-NEM (Kowal *et al.*, 1965) was at 25 °C for up to 30 min, followed by addition of equimolar dithiothreitol to destroy excess NEM. Progress of each modification was monitored by assay of catalytic activity, which was expressed as a percentage of activity before thiol-group modification.

Proteinase-susceptibility of modified aldolases

Labelled aldolase and thiol-modified forms were incubated at 37 °C with either chymotrypsin (Midelfort & Mehler, 1972) (250 mg/ml in 25 mM-Tris/HCl, pH 8) or cathepsin B (20 μg/ml in 20 mM-sodium phosphate/1 mM-dithiothreitol / 1 mM-EDTA, pH 6) (Bond & Offerman, 1981). At various times, sub-samples of each reaction mixture were added to bovine serum albumin (250 μg) in a total volume of 200 μl, and proteins were precipitated by the addition of trichloroacetic acid to 10% (w/v). Overall proteolysis was calculated from the change in acid-insoluble radioactivity during the incubation period. The extent of alteration of the aldolase subunit to smaller polypeptides was measured by dissolving the acid-insoluble pellet in SDS/dithiothreitol at 95 °C, followed by polyacrylamide-gradient-gel electrophoresis (Chandler & Ballard, 1985).

Loading of proteins into erythrocyte ghosts

Labelled aldolase and modified forms were loaded into erythrocyte ghosts by the pre-swell method (Rechsteiner, 1982) with minor modifications (Knowles *et al.*, 1988). The efficiency of loading and the extent to which loaded proteins could be extracted by water lysis of the ghosts (i.e. solubility) were assessed as previously described (Knowles *et al.*, 1988).

Autolysis of proteins in erythrocyte ghosts

Loaded erythrocyte ghosts were incubated at 37 °C in 10 mM-Tris/HCl/150 mM-NaCl, pH 7.4. At various times sub-samples were precipitated with trichloroacetic acid, and proteolysis was calculated from the change in acid-insoluble radioactivity with time.

Fusion of loaded ghosts to tissue-culture cells

HeLa cells were harvested, washed and sedimented, fused to loaded ghosts with inactivated Sendai virus, washed and plated out as described previously (Knowles *et al.*, 1988). After a 6 h incubation to allow re-attachment as monolayer cultures, residual ghosts were lysed and removed, and the cells incubated in Dulbecco's modified Eagle's minimal essential medium plus 5% fetal-bovine serum (Knowles *et al.*, 1988).

Solubility and breakdown of injected proteins

Solubility of injected radioactivity was measured at 6, 24 and 42 h after microinjection by washing the monolayers to remove serum and harvesting the cells by trituration in 1 ml of water. The material harvested with water was freeze-thawed twice and centrifuged at 10000 g for 5 min. Supernatant and pellet fractions were treated with SDS/dithiothreitol, radioactivity was determined, and the proteins were separated on polyacrylamide gradient gels (Chandler & Ballard, 1985).

Breakdown of injected aldolase was measured at 6, 15, 24, 34.5 and 44 h after injection. At each time, medium was removed and the monolayers were washed with 2 × 1 ml of Hanks' salts to remove serum. The radioactivity was measured in these washes. The washed monolayers were then scrape-harvested in two successive 1 ml portions of 10% trichloroacetic acid, after which each combined harvest was centrifuged (10000 *g*, 5 min) to separate acid-soluble and acid-insoluble radioactivity within the cells. The cells were treated with SDS/dithiothreitol and dissociated proteins were separated on gradient gels (Chandler & Ballard, 1985). Polypeptides were detected by fluorography and quantified by liquid-scintillation counting after bands had been cut from the gels (Chandler & Ballard, 1985). The radioactivity remaining on dishes after harvest was measured after addition of 1 ml of 0.5 M-NaOH/0.1% Triton X-100. Since radioactivity was measured in all fractions and washes, any variability between dishes in the number of cells plated or the losses during the washing procedure could be minimized by application of an appropriate correction factor (Chandler & Ballard, 1985).

RESULTS

Loading of modified aldolases into erythrocyte ghosts

Oxidation and inactivation of aldolase by GSSG may have caused a slight decrease in the amount of protein loaded into erythrocyte ghosts from 21% to 15%, but oxidation did not markedly affect the degree of solubility of the loaded protein, which remained at approx. 30% (Table 1). In contrast, NEM-modified aldolase appeared to exhibit progressively greater loading efficiencies and lower solubility than did unmodified aldolase (Table 1). Similar results were seen in most other experiments, but, when aldolase was inactivated with either GSSG or NEM so that 1% or less residual activity remained, the proteins were loaded with greater than 50% efficiency and were less than 10% soluble in the loaded ghosts, indicating extensive binding of the proteins to ghost membranes.

Table 1. Loading of modified aldolases into erythrocyte ghosts

Radiolabelled aldolase was partially inactivated by treatment with GSSG or NEM as described in the Materials and methods section. Variously modified forms of a single preparation of labelled aldolase were loaded into erythrocyte ghosts, after which the proportion of protein loaded and its solubility after water lysis were determined. Two other preparations of aldolase gave similar results.

Modifying agent	Residual activity (%)	Amount loaded (%)	Proportion water-soluble (%)
None	100	21.4	29.9
GSSG	61	16.0	35.2
	17	16.6	29.4
	3	14.7	29.8
NEM	23	23.8	20.3
	11	30.8	15.9

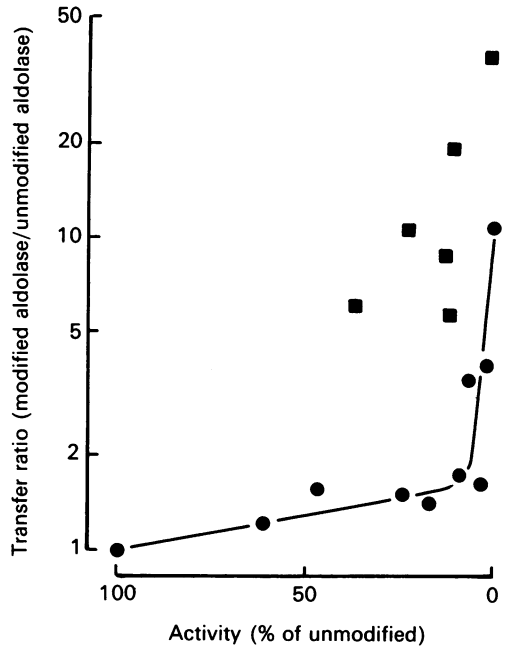

Fig. 1. Differences in transfer of radioactivity to recipient cells with the degree of protein modification

Radiolabelled aldolase, inactivated to different degrees with GSSG (●) or NEM (■), was loaded into erythrocyte ghosts and the ghosts were fused to HeLa cells as described in the Materials and methods section. Radioactivity associated with HeLa cells was measured 6 h after plating. It was expressed as the ratio of the radioactivity transferred from a modified aldolase to the radioactivity transferred from the unmodified aldolase in that experiment. This ratio is plotted against the percentage of catalytic activity retained after modification of the aldolase.

Efficiency of protein transfer and solubility of microinjected protein

Upon fusion with Sendai virus, approx. 0.2% of the ghost-associated radioactivity from unmodified aldolase was transferred to the HeLa cells. Transfer was much greater with the modified aldolases (Fig. 1), since up to 2% of GSSG-treated aldolase and 8% of NEM-modified aldolase could be transferred to HeLa cells. As the extent of enzyme oxidation by GSSG was increased so that residual catalytic activity was decreased, the transfer increased gradually until the residual catalytic activity was approx. 10%. Further oxidation led to much greater transfer of aldolase (Fig. 1). Transfer of NEM-modified forms of aldolase was always greater than that of GSSG-modified forms that retained a similar amount of catalytic activity (Fig. 1).

A decrease in the catalytic activity of aldolase by oxidation with GSSG was associated with a progressive decrease in the solubility of the modified forms after injection. For NEM-modified aldolase essentially all of the radioactivity transferred to the HeLa cells was insoluble (Fig. 2). This inverse correlation between efficiency of transfer and subsequent solubility was also noted when horseradish peroxidase and a range of glycolytic enzymes were microinjected into L6 myoblasts (Hopgood *et al.*, 1986).

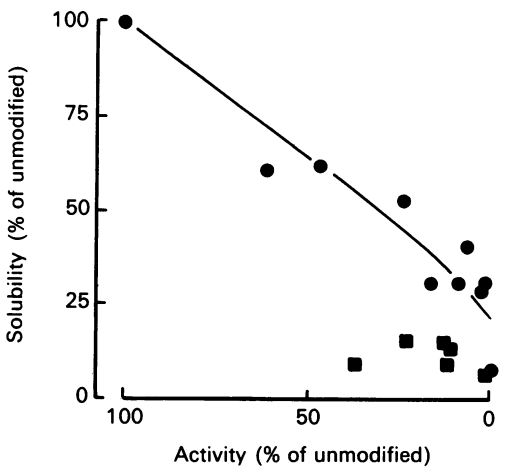

Fig. 2. Effect of aldolase modification on solubility after injection into HeLa cells

HeLa cells were microinjected with aldolase modified by treatment with GSSG (●) or NEM (■). Monolayers were harvested in water 6 h after plating, and soluble and insoluble radioactivity was measured as described in the Materials and methods section. Solubility was expressed relative to that of unmodified aldolase, which averaged 36.2±5.3% (S.E.M.) over five experiments.

Breakdown of the aldolase subunit and its modified forms

Aldolase and its modified forms were subjected to polyacrylamide-gel electrophoresis before and after microinjection. Fluorographic detection of the separated protein subunits indicated that the original ^{3}H-labelled aldolase was an essentially pure 40 kDa monomer. Only traces of lower-molecular-mass bands were detected up to 24 h after fusion (Fig. 3, lanes 1–5). Aldolase that was 97% inactivated by GSSG displayed additional degradation products. A significant product at about 32 kDa was observed by 6 h after fusion, and several other minor radioactive peptides were also present (Fig. 3, lane 8). Modification by NEM resulted in a more complex situation, where a major radioactive peptide of about 38 kDa was found after protein loading into ghosts (Fig. 3, lane 12). This peptide was transferred to HeLa cells by fusion (Fig. 3, lane 13), and other degradation products of approx. 10–14 kDa and 28–32 kDa were also evident. Determination of the radioactivity in these bands (Table 2) revealed some changes in molecular size distribution that were not obvious from fluorography. For the unmodified enzyme, in the first 6 h after microinjection, radioactivity in the 40 kDa aldolase monomer was decreased substantially, from 82 to 49% (Table 2, line 1). Modification of the enzyme resulted in changes in molecular size in the ghosts before microinjection; this was especially evident when NEM treatment was used. The proportion of radioactivity in the 40 kDa protein was further decreased during incubation of the microinjected cells (Table 2).

The radioactivity in the 40 kDa band after microinjection of unmodified aldolase declined, with a first-order rate constant of $-0.023\ h^{-1}$ (Fig. 4). The 40 kDa modified forms of aldolase were all degraded at a rate remarkably similar to this, as indicated by the half-lives shown on Fig. 4.

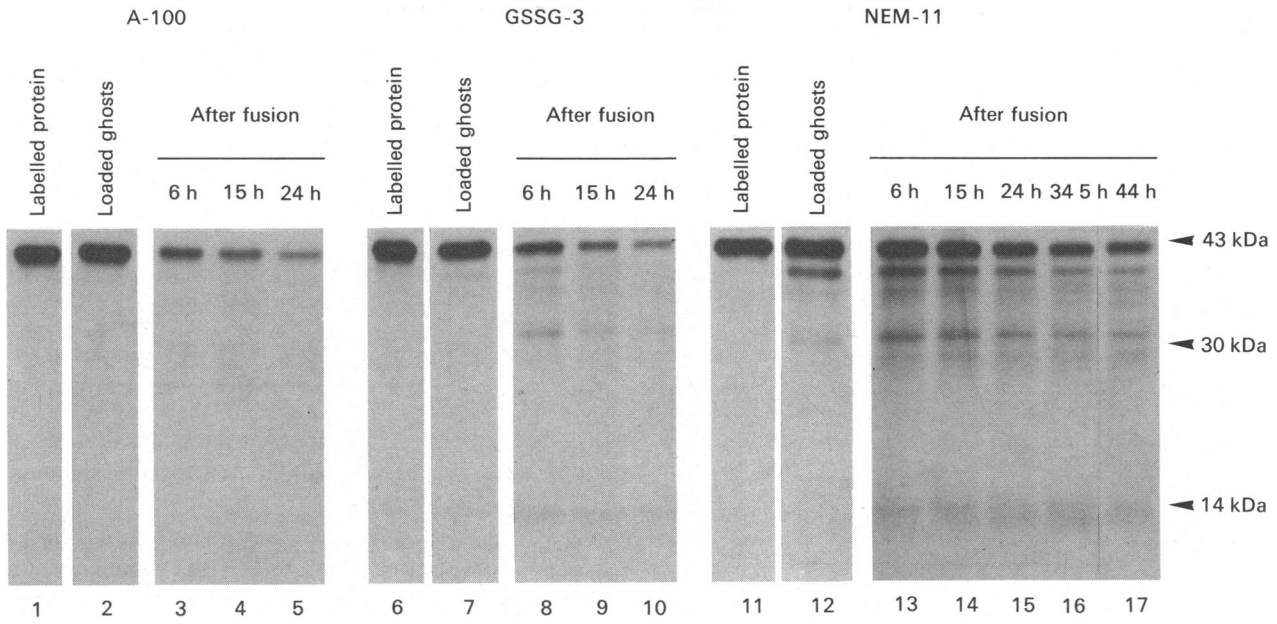

Fig. 3. Autoradiograph after gel electrophoresis of modified aldolases and derived peptides before and after microinjection

Radiolabelled native aldolase (A-100) or aldolase almost completely inactivated by treatment with GSSG (GSSG-3; residual activity 3%) or with NEM (NEM-11; residual activity 11%) was microinjected into HeLa cells via erythrocyte ghosts. Proteins and protein-containing cell fractions were sampled up to 44 h after cell plating, and proteins were separated by gel electrophoresis as described in the Materials and methods section. Radioactivity in peptide bands on the gels was detected by fluorography. Arrowheads show the relative migration of protein standards ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and α-lactalbumin (14 kDa).

Table 2. Aldolase 40 kDa monomer as a proportion of total radioactivity before and after microinjection

Experimental details are as described in Fig. 3 legend, but the gels were sliced into strips and the radioactivity in each strip was determined as described in the Materials and methods section. Values are means ± S.E.M. for data obtained from three wells at the different times for each aldolase.

Modifying agent	Residual activity (%)	Proportion of total radioactivity found at 40 kDa (%)			
		Not injected	Ghosts	Injected cells 6 h	Injected cells 44 h
None	100	82	81	49±3	30±2
GSSG	61	81	75	39±3	25±2
GSSG	3	83	65	33±3	23±2
NEM	23	76	56	35±3	23±3
NEM	11	76	41	36±3	23±1

Susceptibility of modified aldolases to proteolysis in erythrocyte ghosts

To investigate further the degradation of modified and native aldolases, loaded ghosts were incubated for 1–20 h and the loss of trichloroacetic acid-insoluble radioactivity was measured (Fig. 5). Such autolysis in ghosts loaded with unmodified aldolase was very low; only approx. 3% of the protein was hydrolysed to acid-soluble material in 18 h (Fig. 5). Aldolase modified by NEM was up to 8 times more susceptible to autolysis, whereas GSSG-modified enzyme was twice as susceptible (Fig. 5).

Susceptibility of modified aldolases to proteinases *in vitro*

Modified forms of aldolase were incubated with cathepsin B or chymotrypsin *in vitro*. Measurements of trichloroacetic acid precipitability of aldolases during cathepsin B treatment showed that GSSG-oxidized aldolase was hydrolysed less rapidly than the NEM-modified enzyme (Fig. 6). In contrast, the different modified forms were approximately equally susceptible to hydrolysis by chymotrypsin (Fig. 6).

To investigate initial changes made by proteinases to the 40 kDa subunit, the same incubation mixtures used for autolysis were analysed by gel electrophoresis (Fig. 7). Cathepsin B action on unmodified aldolase produced considerable broadening of the 40 kDa monomer band, suggesting the generation of peptides in the range 38–40 kDa (Fig. 7, lane 10). Peptides at about 36 and 32 kDa were detected with the modified aldolases as substrates. Chymotryptic hydrolysis led to the production of other peptides, with small products of about 10 kDa being detected (Fig. 7, lanes 3–7). It was also apparent from the autoradiographs (Fig. 7, lanes 4 and 5, 11 and 12) that the 40 kDa monomer band of GSSG-oxidized aldolase was much decreased after incubation for 2–4 h, whereas measurement of overall hydrolysis by trichloroacetic acid precipitation (Fig. 6, upper panels) indicated little hydrolysis after 1 h. Excising and counting radioactivity in the area of the gel occupied by the 40 kDa monomer (Fig. 6, lower panels) confirmed that the modified forms of the enzyme were indeed extensively altered after only 1 h of incubation.

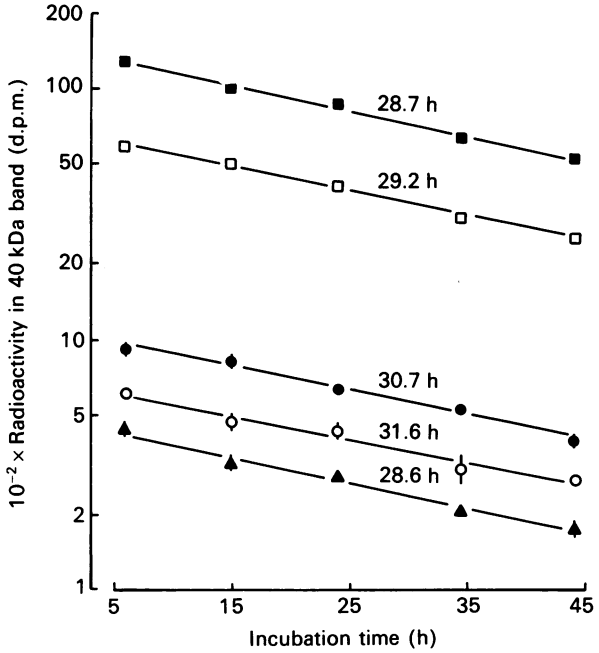

Fig. 4. Effect of aldolase modification on the rate of breakdown of the protein subunit after microinjection into HeLa cells

Radiolabelled native aldolase (▲), or aldolase inactivated with GSSG until the enzyme retained 61% (○) or 3% (●) of catalytic activity, or with NEM until the enzyme retained 23% (□) or 11% (■) of catalytic activity, was loaded into erythrocyte ghosts and transferred into HeLa cells. Cell proteins were separated by gel electrophoresis at periods between 6 h and 44 h after microinjection, and radioactivity in peptides of mobility corresponding to the 40 kDa aldolase subunit was measured as described in the Materials and methods section. Values are means for three dishes, with S.E.M. bars shown where they are sufficiently large. The half-lives of the different forms are indicated.

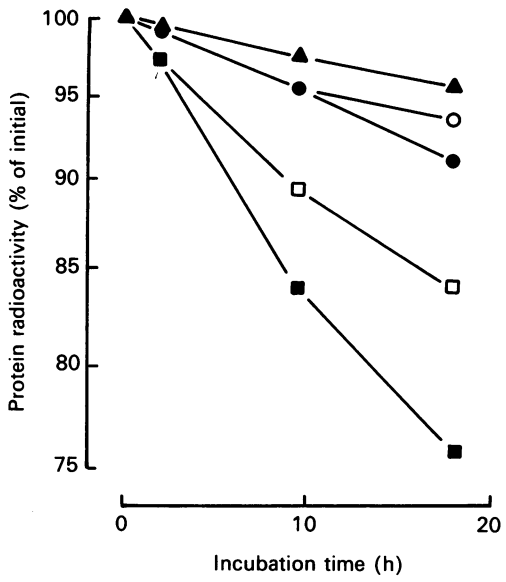

Fig. 5. Autolysis of modified aldolases after loading into erythrocyte ghosts

Erythrocyte ghosts loaded with the proteins described in Fig. 4 were incubated at pH 7.4 and 37 °C. Trichloroacetic acid-insoluble radioactivity was expressed as a percentage of that present at the beginning of the incubation period.

DISCUSSION

The erythrocyte ghost is most suitable as a vehicle for microinjection of proteins when it acts as an inert carrier and does not modify loaded proteins. In practice, radiolabelled proteins show a range of stability inside ghosts. Thus ^{125}I-labelled bovine serum albumin, lysozyme and RNAase A are relatively stable, as judged by electrophoretic mobility and generation of acid-soluble products (Yamaizumi *et al.*, 1978; Neff *et al.*, 1981; Netland & Dice, 1985), whereas other proteins are destroyed at a moderate rate in ghosts incubated at 37 °C (Rechsteiner, 1982). Moreover, Netland & Dice (1985) found that ^{3}H-labelled aldolase was 20% converted into acid-soluble products after incubation of loaded ghosts for 1 h at 37 °C, and ^{125}I-labelled glyceraldehyde-3-phosphate dehydrogenase was almost completely converted into relatively stable, smaller, peptides over this short incubation time. We observe that the erythrocyte ghost is almost inert towards unmodified labelled aldolase, but this is not the case with modified forms of the protein (Fig. 3, Table 2). In particular, NEM-modified aldolase that retained some catalytic activity was found to contain only one-half of its radioactivity as the native protein subunit. We have no information on the nature of the proteinases in erythrocytes that are responsible for the hydrolysis of modified forms of aldolase, but a number of proteinases are present (Tökés & Chambers, 1975; Murakami *et al.*, 1979; Allen & Cadman, 1979; Goldberg & Boches, 1982; Fagan *et al.*, 1986).

The observation of increased transfer of modified aldolases to recipient HeLa cells (Figs. 1 and 2) could perhaps have a trivial explanation. Significant ghost lysis during microinjection plus binding of the released protein to the HeLa-cell surface would result in apparent microinjection of the labelled protein. More avid binding of modified aldolases in proportion to their degree of modification would give the observed relationship. However, we have evidence from related studies that labelled proteins such as enolase (Hopgood *et al.*, 1986), which shows a degree of transfer and solubility similar to that of the modified aldolases, as well as horseradish peroxidase (Knowles *et al.*, 1988), which exhibits low transfer and high solubility, are each transferred to rather less than one-half of the recipient cells. This uneven transfer is not consistent with protein 'sticking' to the cell surface.

A protein altered from its native conformation is generally more susceptible to the action of proteolytic enzymes (Beynon & Bond, 1986), presumably because its structure is partially unfolded. Aldolase is typical in that inactivation of the enzyme by treatment with GSSG or cystine results in greatly increased susceptibility to proteolysis by proteinases, including lysosomal enzymes and the metallo-proteinase meprin (Bond & Offerman, 1981). In addition, acid denaturation of aldolase permits more extensive proteolysis by cathepsin D (Offerman *et al.*, 1983). We observed an increased proteolytic

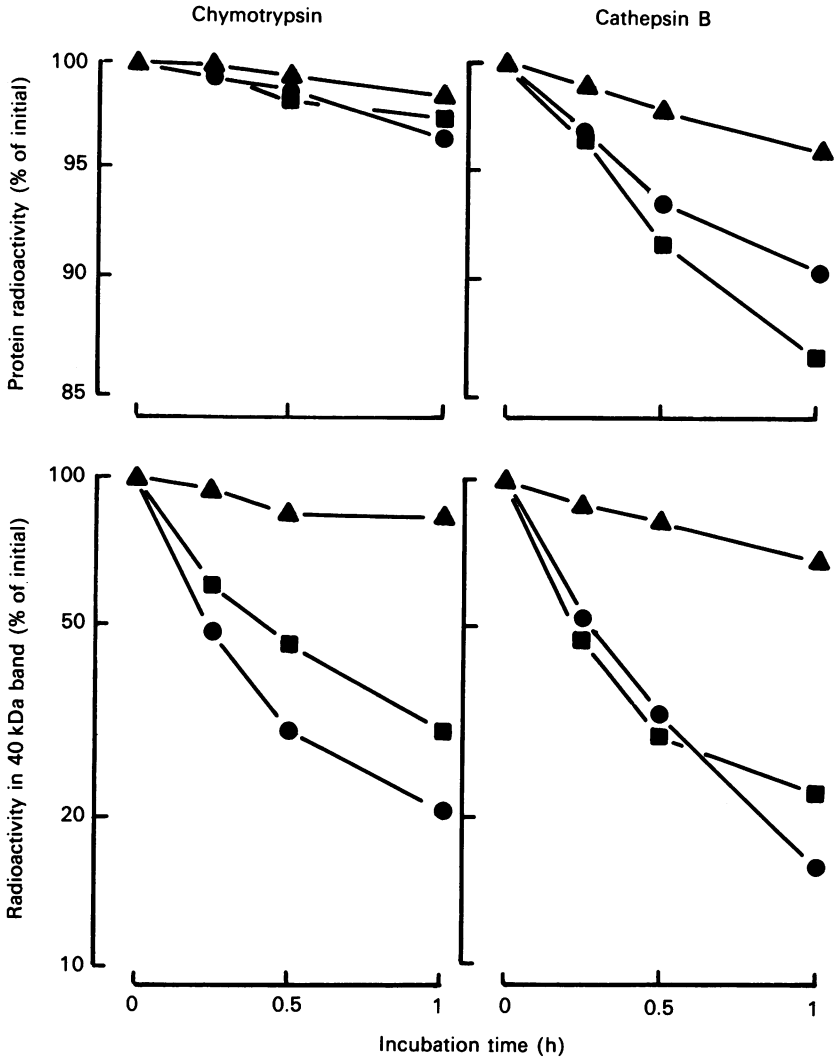

Fig. 6. Susceptibility of modified aldolases to hydrolysis by chymotrypsin and cathepsin B

Radiolabelled native aldolase (▲) or aldolase modified with GSSG (●; residual activity 2%) or NEM (■; residual activity 9%) was incubated at 37 °C with chymotrypsin or cathepsin B as described in the Materials and methods section. Hydrolysis to trichloroacetic acid-soluble products displayed in the top panels was measured and expressed as in Fig. 5. Peptides in the trichloroacetic acid-insoluble fraction were separated by gel electrophoresis, after which radioactivity with the mobility of the 40 kDa aldolase monomer was measured and calculated as a percentage of the starting radioactivity. These percentages are displayed in the bottom panels.

susceptibility of thiol-modified forms of aldolase (Fig. 5). Clearly proteolytic enzymes display selective recognition of particular conformations and exposed bonds of the variously modified aldolase forms.

Certain modified proteins are degraded selectively by intact cells. For example, denatured proteins presented to the exterior of the plasma membrane are endocytosed at a greater rate than native proteins, leading ultimately to more rapid degradation (Ashwell & Morwell, 1974; Tolleshaug *et al.*, 1977). Also, abnormal polypeptides generated inside cells by amino acid substitutions (Knowles *et al.*, 1975; Knowles & Ballard, 1976) or by premature termination with puromycin (McIlhinney & Hogan, 1974) are rapidly degraded. However, the organized degradation systems of HeLa cells clearly make no such distinction between partially inactivated aldolases after microinjection. Using two different types of thiol-group modification and varying the degree of partial inactivation, we found no difference in the rate of breakdown of the aldolase polypeptide chain (Fig. 4).

Recent work from three laboratories provides somewhat comparable results. RNAase A is much more resistant to proteinases *in vitro* than its truncated derivatives RNAase S and S-peptide (Rote & Rechsteiner, 1986). Yet all three species were degraded to acid-soluble products at similar rates after microinjection into HeLa cells (Rote & Rechsteiner, 1986), though at moderately different rates in human diploid fibroblasts (Backer *et al.*, 1983). Rivett (1985) and Rivett & Hare (1986) subjected *Escherichia coli* glutamine synthetase to mixed-function oxidation, and found that enzyme retaining 2% of catalytic activity was hydrolysed 3–4 times faster by trypsin or by the high-molecular-mass liver cysteine proteinase, even though its stability after

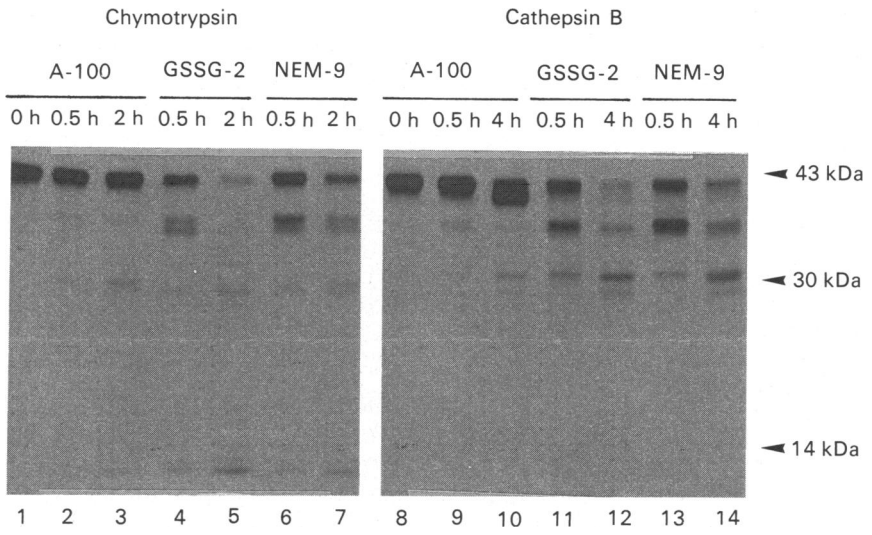

Fig. 7. Electrophoretic separation of peptides generated by the action of chymotrypsin and cathepsin B on modified aldolases

Radiolabelled native aldolase (A-100) or aldolase modified with GSSG (GSSG-2; residual activity 2%) or NEM (NEM-9; residual activity 9%) were incubated at 37 °C with chymotrypsin or cathepsin B. Peptides in the trichloroacetic acid-insoluble fraction were separated by gel electrophoresis and detected by fluorography. Protein standards were as in Fig. 3.

injection into H35 hepatoma cells was unaltered. Interestingly, when the enzyme was oxidized for a longer time such that no catalytically active molecules remained, its susceptibility to proteolysis *in vitro* was further increased and the altered protein was now much less stable after microinjection (Rivett, 1985). A similar situation may apply with aldolase. Thus, when oxidation with GSSG was continued until very little or no catalytic activity remained, transfer of the protein from ghosts to recipient cells was greatly increased (Fig. 1), solubility in recipient cells was much decreased (Fig. 2), and the rate of breakdown to acid-soluble products was increased to 1.6 times above the control rate (results not shown).

The results of the present study argue that proteolytic systems which are known to be selective towards modified protein substrates are unlikely to be involved in the degradation of partially inactivated aldolase after microinjection. Selective mechanisms might include those involving ubiquitin (Ciechanover, 1987), the multicatalytic proteinases (Dahlmann *et al.*, 1985; Ciechanover, 1987) or calpains (Pontremoli & Melloni, 1986). Non-selective engulfment of the microinjected proteins by the autophagic system (Ericsson, 1969) seems more likely to be the degradation pathway employed by the recipient HeLa cell. Sequestration of proteins in bulk from the intracellular milieu into autophagic vacuoles, followed by fusion with primary lysosomes and relatively rapid proteolysis, would result in similar rates of degradation for all sequestered proteins. Data showing that there is significant inhibition of aldolase degradation by lysosomotropic agents such as ammonia (Hopgood *et al.*, 1986) also support the proposition that microinjected aldolase is degraded via the autophagic-lysosomal system. In this respect the regulation of aldolase degradation is similar to that for other microinjected proteins of intracellular origin (McElligott *et al.*, 1985; Rogers & Rechsteiner, 1985; Doherty & Mayer, 1985), rather than to extracellular proteins such as bovine serum albumin (Doherty *et al.*, 1987; Rote & Rechsteiner, 1983) or to foreign proteins such as horseradish peroxidase (Knowles *et al.*, 1988). The degradation of these latter proteins after microinjection is inhibited little or not at all by ammonia, and it is therefore likely that they are degraded extralysosomally. The relatively long half-life of aldolase is also consistent with degradation of the protein by autophagy. In this context, Rogers & Rechsteiner (1985) report a half-life of 77 h for the breakdown of aldolase microinjected into HeLa cells, compared with values ranging from 22 to 101 h for aldolase breakdown measured under various conditions by biosynthetic labelling and the 30 h half-life in the present study. These rates are slower than those reported for bovine serum albumin (half-life 16–20 h) (Doherty *et al.*, 1987; Rote & Rechsteiner, 1983) or horseradish peroxidase (half-life approx. 15 h) (Knowles *et al.*, 1988), which are probably not degraded by the autophagic-lysosomal system.

It was known from previous reports, and confirmed here, that GSSG and NEM inactivate and destabilize aldolase and make the protein more susceptible to proteolysis *in vitro*. The present microinjection studies indicate that the proteolytic systems of the cell do not recognize the difference between native and modifed forms of aldolase and do not degrade selectively the more denatured forms. We suggest that the aldolase forms are all degraded by a random autophagic process involving the lysosomal system.

We thank staff at the Red Cross Blood Transfusion Service, Adelaide, for their continued support in providing blood samples.

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Received 11 January 1988/10 June 1988; accepted 28 June 1988