Enzyme Inactivation via Disulphide–Thiol Exchange as Catalysed by a Rat Liver Membrane Protein

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1. The inactivation of cytosol enzymes by a rat liver membrane protein was studied with crude microsomal fraction, plasma membranes or a partially purified preparation of inactivation factor. 2. Complete inactivation of ^{125}I -labelled glucose 6-phosphate dehydrogenase (EC 1.1.1.49) by membranes did not result in any detectable change in molecular weight when the products were analysed by gradient polyacrylamide-gel electrophoresis. 3. Inactivation of radioactive enzyme was not accompanied by extensive binding to the membrane surface. The maximum extent of binding was 15% of the total enzyme labelled, and bound radioactivity was released only slowly, mainly as trichloroacetic acid-insoluble material. 4. Treatment of membranes with dithiothreitol destroyed the inactivation capacity, whereas the thiol-alkylating agent iodoacetamide had no effect. Partial restoration of the inactivation capacity of reduced membranes after exposure to air was prevented by membrane alkylation with iodoacetamide. 5. Modification of enzyme thiol groups during inactivation was determined by measuring a decrease in iodoacetamide-reactive groups in purified glucose 6-phosphate dehydrogenase. 6. Incubation of membrane-inactivated glucose 6-phosphate dehydrogenase with dithiothreitol resulted in a partial restoration of enzyme activity. 7. As a result of these experiments it is concluded that inactivation proceeds by a disulphide–thiol exchange mechanism. The proposal that this reaction could be involved in the initial step of enzyme degradation is discussed.

Protein denaturation has been proposed as the rate-limiting step in enzyme degradation (Hopgood & Ballard, 1974; Ballard & Hopgood, 1976; Goldberg & St. John, 1976), with the loss of catalytic activity as the initial event in a sequential set of reactions (Ballard *et al.*, 1974). In the preceding paper we reported further studies on the distribution and partial purification from rat liver microsomal fraction of a membrane protein capable of inactivating cytosol enzymes at rates approximately proportional to their degradation rates *in vivo* (Francis & Ballard, 1980). However, despite a number of proposals (Dean, 1975; Bond, 1976; Bohley *et al.*, 1977), the means by which enzymes could be selectively inactivated *in vivo* is not clear.

Previous investigations on the microsomal inactivation factor implicated disulphides in the reaction, possibly through a disulphide–thiol exchange (Ballard & Hopgood, 1976). Other studies have shown that a number of enzymes are inactivated by L-cystine or disulphides (Bradshaw *et al.*, 1967; Ernest & Kim, 1973; Federici *et al.*, 1978; Shimazu *et al.*, 1978), and in all cases it was concluded that

formation of mixed disulphides was involved. In the present paper we report evidence that the microsomal inactivation factor operates by a mechanism involving exchange between enzyme thiol groups and a disulphide associated with the factor. Part of the work described has been presented in preliminary form (Ballard *et al.*, 1977).

Materials and Methods

Chemicals

Bolton and Hunter reagent [*N*-succinimidyl 3-(4-hydroxy-5- ^{125}I iodophenyl)propionate] and iodo[1- ^{14}C]acetamide were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.); all enzymes unless otherwise stated, cofactors, nucleotides and dithiothreitol were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); iodoacetamide was from Calbiochem (La Jolla, CA, U.S.A.).

Enzyme measurements

Assays for the following enzymes were carried out at the temperatures indicated, without further modi-

fications: phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32), 37°C (Ballard & Hanson, 1969); glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 25°C (Kornberg & Horecker, 1955); aldolase (EC 4.1.2.13), 25°C (Clark *et al.*, 1973). A unit of enzyme is that amount of activity that catalyses either the formation of 1 μ mol of product or the removal of 1 μ mol of substrate/min.

Substrate measurements

NADP⁺ was assayed by using a modification of the glucose 6-phosphate dehydrogenase assay method (Kornberg & Horecker, 1955) in which NADP⁺ was omitted and 0.15 unit of yeast glucose 6-phosphate dehydrogenase/ml of reaction mixture was included.

NADPH was determined by measuring the glutathione reductase-catalysed oxidation of NADPH by oxidized glutathione at 25°C (Ansorge *et al.*, 1973). Each cuvette contained 50 nmol of oxidized glutathione and 5 units of glutathione reductase in a final volume of 1 ml.

Standard assay for the inactivation factor

Incubation mixtures contained 90 μ l of rat liver cytosol (Francis & Ballard, 1980), 50 μ l of freshly prepared 10 mM-L-cystine, pH 8, 10 μ l of 0.5 M-Tris/HCl buffer, pH 8, 10 μ l of 1% Triton X-100 plus sample and other additions in a total volume of 250 μ l. Samples were taken after incubation at 37°C for various time periods and cooled in ice for subsequent measurement of enzyme activities. Inactivation capacity is expressed as a percentage of initial activity remaining, or the half-time for enzyme inactivation where first-order kinetics are observed (Francis & Ballard, 1980).

Preparation of membranes

Plasma membranes and microsomal fraction (referred to below simply as 'microsomes') were prepared from starved rats as reported previously (Francis & Ballard, 1980).

Preparation of ¹²⁵I-labelled glucose 6-phosphate dehydrogenase

Purified rat liver glucose 6-phosphate dehydrogenase (Francis & Ballard, 1980) was labelled with ¹²⁵I to high specific radioactivity by using the Bolton and Hunter reagent [*N*-succinimidyl 3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate, specific radioactivity 1375 Ci/mmol of the mono-iodo ester] (Bolton & Hunter, 1973). ¹²⁵I-labelled glucose 6-phosphate dehydrogenase recovered from the Sephadex G-50 gel-filtration step was stored frozen.

Partially purified inactivation factor

Inactivation factor was purified 115-fold from microsomes to give a specific activity of 6330 m-units/mg of protein (Francis & Ballard, 1980).

Gradient polyacrylamide-gel electrophoresis

Electrophoresis on linear 4–26% polyacrylamide gradient gel slabs (82 mm × 82 mm × 6 mm) in glass cells was performed on the Gradipore apparatus (Gradient Pty. Ltd., Lane Cove, N.S.W., Australia). Samples were loaded into the plastic sample spacer, and after electrophoresis appropriate parallel strips of gel were sectioned for radioactivity determination or stained for lactate dehydrogenase (Gabriel, 1971) or glucose 6-phosphate dehydrogenase activity (Harrison, 1974).

Thiol-group titration with iodo[1-¹⁴C]acetamide

Iodo[1-¹⁴C]acetamide (specific radioactivity 57 mCi/mmol, 50 μ Ci) was transferred quantitatively to a glass vial with 1 ml of redistilled ethanol, sealed with a septum cap and stored with desiccant at –15°C. Required volumes were removed by syringe and evaporated to dryness with a stream of high-purity N₂, and dissolved in 8 M-urea before being added to assays as described in the legend to Fig. 2.

Replication of experiments

The data reported in Tables and Figures are for individual experiments. Essentially similar results were obtained in repeats of every experiment.

Results

Investigation of a role for proteolysis in the inactivation reaction

One of the most likely reactions by which enzymes could be inactivated is proteolytic cleavage. Earlier studies with ³H-labelled phosphoenolpyruvate carboxykinase (GTP) suggested that microsomal inactivation proceeded via a sequence of events not involving an initial proteolytic step (Ballard *et al.*, 1974). These findings have been extended by using ¹²⁵I-labelled glucose 6-phosphate dehydrogenase and a plasma-membrane source of inactivation capacity. The latter has been shown to have similar properties to the microsomal fraction, but with higher specific activity and less contamination by other membranes (Francis & Ballard, 1980). No indication of molecular-weight changes were evident on analysis of the reaction products on gradient polyacrylamide gels after almost complete inactivation of the labelled substrate (Fig. 1). The leading radioactivity peak that does not correspond to an enzyme activity band is located where the inactive glucose 6-phosphate dehydrogenase monomer would be expected. This peak does not show any change during enzyme inactivation. The main peak of radioactivity is coincident with the major glucose 6-phosphate dehydrogenase band and probably represents the 110 000-mol.wt. dimer. Inactivation was associated with a consistent broadening of this peak,

possibly reflecting a change in molecular shape as a result of denaturation or intramolecular rearrangement. No radioactivity was associated with the

Fig. 1. Analysis of inactivated ^{125}I -labelled glucose 6-phosphate dehydrogenase by gradient polyacrylamide-gel electrophoresis

^{125}I -labelled glucose 6-phosphate dehydrogenase (0.42 unit, 5.8×10^4 c.p.m.) was incubated with 1 mM-L-cystine, pH 8, in the presence (●) or absence (○) of plasma membranes (0.160 mg of protein). After 1 h, 8% and 100% of the initial activity remained respectively. NADP^+ was then added to 1 mM and the tubes were cooled to 4°C . Samples from both tubes were analysed at 4°C by electrophoresis on a polyacrylamide-gradient-gel slab for 16 h at 100 V in the presence of glucose 6-phosphate dehydrogenase and lactate dehydrogenase markers. Parallel strips of gel were cut for enzyme staining and for the determination of radioactivity. Details of plasma membrane and ^{125}I -labelled glucose 6-phosphate dehydrogenase preparations are given in the Materials and Methods section. In the gel the solid and stippled bars represent positions of glucose 6-phosphate dehydrogenase and lactate dehydrogenase markers respectively.

minor band of enzyme activity at the position corresponding to that expected for the enzyme tetramer. Analysis of plasma-membrane-inactivated ^{125}I -labelled glucose 6-phosphate dehydrogenase by Sephadex G-200 chromatography also failed to show any change in the pattern of radioactivity after prolonged incubation, other than a minor increase at the void volume.

Ability of NADP^+ and its structural derivatives to protect against inactivation

Use of low concentrations of NADP^+ to stabilize purified glucose 6-phosphate dehydrogenase during the inactivation assay led to the observation that at NADP^+ concentrations of 1 mM or higher the microsomal inactivation capacity was completely inhibited.

Preliminary tests showed that the substrate glucose 6-phosphate was much less effective in protecting cytosol glucose 6-phosphate dehydrogenase than was the coenzyme and that the substrate analogue 2-deoxyglucose 6-phosphate actually stimulated inactivation. Although NADP^+ is a coenzyme and substrate for the enzyme, it might still exert its protective effect by interacting directly with the inactivation factor. Consequently the ability of NADP^+ to protect cytosol enzymes for which it was not a substrate was tested (Table 1). Phosphoenolpyruvate carboxykinase (GTP) and aldolase as well as glucose 6-phosphate dehydrogenase were protected against inactivation by either microsomes or partially purified inactivation factor. To extend these findings and determine whether the effect was specific for NADP^+ , the capacity of a number of structural derivatives of the coenzyme to inhibit the inactivation process was investigated (Table 2). All the pyridine nucleotides gave substantial protection. The minor quantitative variations between deriva-

Table 1. *Ability of NADP^+ to protect cytosol enzymes against inactivation*

Rat liver cytosol was incubated at 37°C in the presence of 2 mM-L-cystine, 30 mM-Tris/HCl buffer, pH 8, 0.5% Triton X-100 with or without 1.0 mM- NADP^+ and either no source of inactivation factor, 1 mg of microsomal protein or $20\mu\text{g}$ of partially purified factor. Samples were taken at 0, 15, 30, 45, 60, 90 and 180 min for the measurement of residual enzyme activity. The half-time of inactivation was calculated for each enzyme as indicated in the Materials and Methods section.

Enzyme	Inactivation factor	Half-time for inactivation (min)	
		- NADP^+	+ NADP^+
Glucose 6-phosphate dehydrogenase	None	360	Stable
	Microsomes	17	1230
	Purified	18	290
Phosphoenolpyruvate carboxykinase (GTP)	None	690	440
	Microsomes	21	310
	Purified	17	400
Aldolase	None	Stable	Stable
	Microsomes	87	Stable
	Purified	88	Stable

Table 2. *Inhibition of the inactivation reaction by nicotinamide derivatives*

Each compound was added to give a final concentration of 1 mM in the standard inactivation assay together with partially purified inactivation factor (6 μ g of protein). Samples were taken at 0, 15, 30, 45 and 60 min for the determination of the half-times for inactivation.

Compound	Half-time for inactivation (min)	
	Glucose 6-phosphate dehydrogenase	Phosphoenolpyruvate carboxykinase (GTP)
No inactivation factor	Stable	Stable
No addition	35	26
NADP ⁺	170	140
NADPH	380	160
NAD ⁺	110	120
NADH	140	180
Acetylpyridine analogue of NAD ⁺	100	140
Nicotinamide	42	30
Nicotinic acid	44	37
Adenine	31	35
AMP	54	71
ADP	61	40
FAD	110	—

Table 3. *Modification of the microsomal inactivation reaction by NADP⁺*

Microsomes were preincubated in the absence (A) or presence (B) of 2 mM-NADP⁺ dissolved in 50 mM-Tris/HCl buffer, pH 8.0, at 37°C for 75 min, by which time no unmodified NADP⁺ could be detected by using yeast glucose 6-phosphate dehydrogenase. Samples of these microsomes were assayed for their ability to inactivate cytosol enzymes as described in the Materials and Methods section. Other portions of the mixtures were centrifuged for 6 $\times$ 10⁶ g-min at 4°C, and the supernatants were removed and kept on ice (supernatants A and B). The pellets were suspended in 50 mM-Tris/HCl buffer, pH 8, centrifuged and the supernatants discarded. Portions of the washed pellets (pellets A and B) were resuspended in supernatants A or B and assayed for inactivation capacity.

Treatment	Enzyme activity remaining after 30 min (%)	
	Glucose 6-phosphate dehydrogenase	Phosphoenolpyruvate carboxykinase (GTP)
Preincubation A	42	36
Preincubation B	97	92
Pellet A + supernatant A	30	34
Pellet A + supernatant B	96	90
Pellet B + supernatant A	91	64
Pellet B + supernatant B	100	96

tives were similar for both glucose 6-phosphate dehydrogenase and phosphoenolpyruvate carboxykinase (GTP). More variability was observed among the remaining compounds, with the free base being less effective than the mono- or di-phosphate ester of adenosine. FAD gave a similar degree of protection against inactivation of glucose 6-phosphate dehydrogenase to that observed for the pyridine nucleotides.

Since the above results suggested that NADP⁺ may interact directly with the inactivation factor, it was noteworthy that preincubation of the cofactor with microsomes destroyed its capacity to act as a substrate in the yeast glucose 6-phosphate dehydrogenase reaction (line 1 versus line 2, Table 3). For these experiments the yeast enzyme was used simply

as an assay for NADP⁺. The nature of the interaction between NADP⁺ and microsomes was further investigated by preincubating these reagents together until no NADP⁺ could be detected (Table 3). Such treatment destroyed the ability of microsomes to inactivate either glucose 6-phosphate dehydrogenase or phosphoenolpyruvate carboxykinase (GTP) in an analogous way to that shown in Table 2 for the purified inactivation factor. However, after the microsomes were removed from the mixtures by centrifugation, mixing experiments showed that supernatants retained the ability to protect the enzymes from inactivation (see pellet A plus supernatant B, Table 3). Also, the NADP⁺-treated microsomes had been modified to such an extent that they were unable to carry out the inactivation reaction

even in the absence of NADP⁺ (pellet B plus supernatant A, Table 3). It should also be noted that if NADPH was substituted for NADP⁺ in this experiment the reduced coenzyme was modified such that it was no longer a substrate for glutathione reductase. Whatever the modification involved, the absorbance at 340nm characteristic of NADPH was not affected.

Effect of modification of membrane thiol groups on the inactivation reaction

Since a proteolytic reaction is apparently not involved in enzyme inactivation, a conformational change or the binding of ligands is presumably implicated. As noted above, one constant feature of the reaction has been a stimulation by disulphides and an inhibition by thiols. When liver plasma membranes were treated with dithiothreitol to reduce disulphides and subsequently the dithiothreitol was removed, the modified membranes lost their ability to inactivate glucose 6-phosphate dehydrogenases even in the presence of L-cystine (Table 4). However, addition of iodoacetamide to alkylate available thiol groups had no effect on inactivation capacity unless the membranes had been reduced previously by dithiothreitol. Partial restoration of the inactivation activity of reduced membranes occurred after re-incubation of the mixture in air. This was prevented by alkylation of the dithiothreitol-treated membranes with iodoacetamide, a treatment that would also prevent oxidation of thiols.

Binding of ¹²⁵I-labelled glucose 6-phosphate dehydrogenase to plasma membranes

Plasma-membrane binding studies with ¹²⁵I-labelled glucose 6-phosphate dehydrogenase suggested that inactivation was not simply the result of

a non-specific membrane-binding phenomenon. Although enzyme inactivation was associated with greater binding (Table 5), it amounted to only 15% of the total radioactivity present in ¹²⁵I-labelled glucose 6-phosphate dehydrogenase even under conditions where the enzyme was 100% inactivated. Reduction and alkylation of membranes with dithiothreitol followed by iodoacetamide destroyed the inactivation capacity (Table 5) and also markedly diminished binding of labelled enzyme to the plasma membranes. Incubation of labelled enzyme with erythrocyte 'ghosts', a condition that does not lead to inactivation (Francis & Ballard, 1980), gave no binding to the membranes.

In an attempt to analyse the binding reaction further, ¹²⁵I-labelled glucose 6-phosphate dehydrogenase was first bound to plasma membranes and subsequently re-incubated under inactivating conditions with unlabelled enzyme. A low rate of exchange between unlabelled and labelled enzyme was indicated because bound ¹²⁵I-labelled enzyme was released only slowly. However, released radioactivity was mainly as trichloroacetic acid-insoluble material, implying slight proteolysis. The addition of L-cystine to stimulate microsomal inactivation of cytosolic enzymes (Ballard & Hopgood, 1976) had no significant effect on the rate of release of labelled enzyme.

Analysis of substrate thiol groups after inactivation

The above results taken together support our earlier view that inactivation may involve an exchange reaction between disulphide compounds and free thiol groups on the substrate (Ballard & Hopgood, 1976). Consistent with this mechanism would be an oxidation of at least one thiol group during the inactivation reaction. To test directly whether the

Table 4. *Effects of modification to plasma membranes on glucose 6-phosphate dehydrogenase inactivation*

Plasma membranes from rat liver were either reduced by treatment with 50 mM-dithiothreitol or alkylated by incubation in 0.1 M-iodoacetamide, pH 8. The membranes were subsequently pelleted by centrifugation for 6×10^6 g-min. To produce reduced and alkylated membranes, the pelleted membranes after dithiothreitol treatment were treated with 0.1 M-iodoacetamide and pelleted. Oxidation of variously treated membranes was attempted by incubation in air at 25°C for 2 h. All membrane fractions were eventually suspended in 50 mM-Tris/HCl buffer, pH 8, at concentrations of 0.4 mg of protein/ml (Expt. 1) or 0.2 mg of protein/ml (Expt. 2). Inactivation capacity was measured by using the standard assay. n.d., Not determined.

Membrane treatments	Expt. 1	Expt. 2
	(% of enzyme remaining at 40 min)	(% of enzyme remaining at 120 min)
Control (no plasma membrane)	100	100
No treatment	24	3
Reduced	100	69
Alkylated	26	3
Reduced, alkylated	100	100
Reduced, oxidized	n.d.	37
Reduced, alkylated, oxidized	n.d.	100
Alkylated, oxidized	n.d.	4

Table 5. Relationship between inactivation of ^{125}I -labelled glucose 6-phosphate dehydrogenase and binding to plasma membranes

^{125}I -labelled glucose 6-phosphate dehydrogenase (2.2 units of activity, 6.8×10^5 c.p.m.) was incubated with liver plasma membranes (0.5 mg of protein/ml) in 50 mM-Tris/HCl buffer, pH 8, at 37°C for 3 h. Where indicated NADP $^+$ was added at 1 mM and cystine at 2 mM concentrations. The treated membranes were prepared as described in the legend to Table 4. Binding was determined after centrifugation of membranes through 35 vol. of 0.25 M-sucrose in 10 mM-Tris/HCl buffer, pH 8, for 6×10^6 g-min. Prelabelled membranes were incubated and pelleted as described above. The membrane pellets were resuspended in 50 mM-Tris/HCl buffer, pH 8, unlabelled glucose 6-phosphate dehydrogenase (2.2 units of activity) was added and the mixtures were incubated for a further 3 h at 37°C . Trichloroacetic acid-soluble radioactivity (degraded enzyme) and insoluble radioactivity (released enzyme) were measured in separate portions of each incubation mixture after the addition of trichloroacetic acid to give a final concentration of 10%. Zero-time values for binding and release of radioactivity have been subtracted. Glucose 6-phosphate dehydrogenase activity remaining after 3 h was determined. For prelabelled membranes this refers to the incubation with unlabelled enzyme. n.d., Not determined.

Membrane treatments	Addition	Enzyme activity remaining (% after 3 h)	Enzyme bound (% at 3 h)	Enzyme degraded (% after 3 h)	Enzyme released (% after 3 h)
None	None	3	9.8	2.33	n.d.
None	NADP $^+$	40	3.0	1.64	n.d.
None	Cystine	2	11.2	2.03	n.d.
Reduced, alkylated	None	100	2.7	0.64	n.d.
Prelabelled	None	5	n.d.	1.92	15.4
Prelabelled	Cystine	4	n.d.	2.50	16.6

Fig. 2. Titration of thiol groups after inactivation

Incubation mixtures contained: purified rat liver glucose 6-phosphate dehydrogenase (4.1 units, $60 \mu\text{g}$ of protein), partially purified inactivation factor ($3.8 \mu\text{g}$ of protein) and 0.1 mM-NADP $^+$ (○); enzyme and inactivation factor at the same concentrations as above but 10 mM-NADP $^+$ (▲); partially purified inactivation factor as above, no enzyme and NADP $^+$ at 0.1 mM (△); glucose 6-phosphate dehydrogenase (4.1 units), 0.1 mM-NADP $^+$ but no partially purified inactivation factor (●). In (b) 1 mM-L-cystine was also present. After incubation at 37°C for 3 h (a) or 90 min (b), iodo[1- ^{14}C]acetamide ($1.4 \mu\text{Ci}$, 24.6 nmol) was added and samples were adjusted to 8 M-urea, 1 mM-EDTA and pH 8 before incubation for a further 1 h at 37°C . Samples were then diluted with an equal volume of 0.1 M-Tris/HCl buffer, pH 8, containing 0.1 M-KCl, 8 M-urea and 1 mM-EDTA before chromatography on a $60 \text{ cm} \times 0.65 \text{ cm}^2$ Sepharose CL-6B column equilibrated with the above buffer. Fractions of volume 1.0 ml were collected and radioactivity was determined.

process was associated with a modification of substrate thiols, we looked for a diminution of ^{14}C -labelled iodoacetamide-reactive groups in reaction products. Partially purified inactivation factor and

highly purified rat liver glucose 6-phosphate dehydrogenase were used for this experiment. This allowed resolution of the titrated products by Sepharose CL-6B gel filtration under denaturing

conditions with far less interference from non-specific proteins than if a crude inactivation factor had been used (Fig. 2). Inactivation of glucose 6-phosphate dehydrogenase was clearly associated with a decrease in iodo[1- 14 C]acetamide incorporation into the major peak between fractions 16 and 25 (Fig. 2a). Analogous results were also observed in the presence of L-cystine (Fig. 2b). However, in the absence of L-cystine, inactivation resulted in a greater quantity of radioactivity being eluted in the vicinity of the void volume. The failure to label the inactivation factor in this experiment is consistent with the above studies, where membrane inactivation capacity was unaffected by iodoacetamide treatment without prior reduction by dithiothreitol. In the experiment depicted in Fig. 2(a), the incubation of

glucose 6-phosphate dehydrogenase alone resulted in 76% of the initial activity remaining after 3 h whereas in the presence of inactivation factor this was only 20%. The inclusion of 1 mM-NADP⁺ (Fig. 2a, $\blacktriangle$) diminished the degree of inactivation so that 40% of the activity remained. The amounts of iodoacetamide bound in the experiments shown in Fig. 2(a) for the three conditions were respectively 0.84, 0.37 and 0.52 nmol/nmol of glucose 6-phosphate dehydrogenase monomer when inactivation factor was present, absent or added with 1 mM-NADP⁺.

Reversal of the inactivation reaction by dithiothreitol

If the inactivation reaction proceeds by an initial disulphide-thiol exchange, then treatment of inactivated enzyme with a reducing agent might result in restoration of activity. This experiment was carried out by first inactivating purified rat liver glucose 6-phosphate dehydrogenase by microsomes in the presence of L-cystine. Subsequently re-activation of enzyme was attempted in the presence of 20 mM-dithiothreitol and 5 mM-NADP⁺ (Fig. 3). The control treatments showed a time-dependent activation that decreased in magnitude throughout the incubation period. Microsome-inactivated glucose 6-phosphate dehydrogenase showed a much larger re-activation. Complete re-activation was observed for early time periods, but decreased markedly with prolonged incubation. Essentially similar results were obtained when the inactivation reaction was carried out in the absence of L-cystine, except that less re-activation was obtained when more than 75% of the initial activity had been lost.

Discussion

The degradation of phosphoenolpyruvate carboxykinase (GTP) in liver homogenates was shown previously to proceed by a pathway involving a sequential loss of catalytic activity, antibody reactivity and solubility (Ballard *et al.*, 1974). Subsequently microsomal membranes were shown to catalyse the loss of catalytic activity, a process that was stimulated by disulphides and retarded by free thiols (Ballard & Hopgood, 1976). We have presented evidence that inactivation capacity is not a general property of biological membranes brought about by non-specific binding (Francis & Ballard, 1980). The present experiments with 125 I-labelled glucose 6-phosphate dehydrogenase failed to indicate the involvement of an initial proteolytic cleavage in the inactivation process. This is in agreement with earlier work with 3 H-labelled phosphoenolpyruvate carboxykinase *in vivo* and *in vitro* (Ballard *et al.*, 1974). Furthermore, proteinase inhibitors were found to have no effect on the microsomal inactivation reaction (Ballard & Hopgood, 1976). Together

Fig. 3. Thiol-mediated re-activation of inactivated enzyme

Purified rat liver glucose 6-phosphate dehydrogenase (2.5 units) was initially incubated at 37°C with 2 mM-L-cystine at pH8 in the absence (a) or the presence (b) of microsomes (0.5 mg of protein). Portions were removed at the times indicated for dilution with an equal volume of either 50 mM-Tris/HCl buffer, pH8 (O), or the same buffer containing 40 mM-dithiothreitol and 10 mM-NADP⁺ at 0°C. The latter mixture was re-incubated at 37°C for 30 min, 90 min and 150 min before assay of proteins for glucose 6-phosphate dehydrogenase activity (●).

these results argue that proteolysis is not involved in this process.

The search for a mechanism of the inactivation reaction has centred around the susceptibility of the process to thiols or reagents that interact with them. Previously it was proposed that inactivation might proceed by an exchange reaction between added disulphides and free thiol groups on the enzymes (Ballard & Hopgood, 1976). Indeed low-molecular-weight disulphides appear to mimic the reaction as well as stimulating inactivation by membranes. It would be expected that, in the absence of added low-molecular-weight disulphides, membrane disulphide groups would be essential for the reaction. This concept is supported by the demonstration that membranes previously treated with disulphide-reducing agents do not carry out the inactivation process (Table 4). To explain the results of these experiments we propose the inactivation mechanism depicted diagrammatically in Scheme 1. In this scheme the initial process is an exchange reaction between membrane disulphide groups and enzyme thiol groups, leading to the creation of either intra-enzyme disulphides or enzyme-membrane mixed disulphides. The inactivating factor may have a disulphide group involved in its active site, since the partially purified factor is not stimulated significantly by L-cystine in its inactivation of purified glucose 6-phosphate dehydrogenase. In agreement with the above reaction scheme is the finding that inactivation of glucose 6-phosphate dehydrogenase is associated with a progressive loss of available thiol groups (see Fig. 2). The reversal of inactivation by thiols is also consistent with the proposed mechanism. This re-activation of inactivated enzyme by dithiothreitol suggests that the loss of thiol groups as

measured in the iodo[1- 14 C]acetamide titration experiments results from formation of disulphide linkages. At least with glucose 6-phosphate dehydrogenase the eventual linkages are surely intra-enzyme, because we could not detect an increase in enzyme molecular weight that would accompany inter-enzyme or enzyme-factor bonds. Essentially the same results were obtained in the presence or absence of L-cystine, a result that argues against an initial formation of a mixed disulphide with a low-molecular-weight compound. Furthermore, in experiments with L-[35 S]cystine and microsomes we were unable to show isotopic exchange with purified rat liver glucose 6-phosphate dehydrogenase. The basis for L-cystine stimulation is envisaged in Scheme 1 as the regeneration of membrane disulphide groups after they have been reduced by reaction with the enzyme. Alternatively L-cysteine generated from L-cystine could facilitate rapid exchange and thus release of the products from a transient membrane-enzyme mixed disulphide. An L-cysteine-membrane disulphide formed in this way could then undergo internal exchange to regenerate the membrane disulphide.

The operation of such a scheme *in vivo* would be dependent on a system for reoxidation of the membrane thiol groups, otherwise a steady state cannot occur. The normally high ratio of thiol groups to disulphide groups in cells indicated by the high ratio of reduced glutathione to oxidized glutathione (Eggleson & Krebs, 1974) does not take into account the content of protein thiol groups, protein disulphide groups or protein-glutathione mixed disulphides. The mixed disulphides are largely associated with microsomes (Isaacs & Binkley, 1977a), perhaps implying the presence of a disulphide-generating

Scheme 1. *Proposed mechanism for enzyme inactivation by membrane disulphides*

1, Active enzyme is envisaged as being oxidized to an inactive form by membrane disulphides in the presence of an inactivating factor. 2, Membrane thiols are reoxidized by a source of disulphide indicated here as GSSG (oxidized glutathione) and catalysed by a second enzyme. 3, The inactivated substrate enzyme may be re-activated by thiols, or owing to thermal instability *in vitro* may become irreversibly denatured. 4, The site of action of iodoacetamide on membranes is indicated.

system within this fraction. The microsomal mixed-function oxidase is one possibility for this generating system. These enzymes have been proposed to provide the oxidative step in a cycle leading to the H_2O_2 -dependent oxidation of reduced glutathione to oxidized glutathione by glutathione peroxidase (Isaacs & Binkley, 1977b) or the formation of cystamine from cysteamine (Ziegler & Poulsen, 1977). In the latter case cystamine may then undergo exchange with reduced glutathione, forming oxidized glutathione. Scheme 1 envisages re-oxidation of the membrane thiol groups by oxidized glutathione. However, the localization of cystamine on the microsomal membrane and its capacity to stimulate the inactivation reaction may indicate a direct involvement *in vivo*.

Disulphide exchange reactions have been proposed to be involved in the regulation of a number of enzymes (Pontremoli & Horecker, 1970; Ernest & Kim, 1973; Shimazu *et al.*, 1978; Buckley & Milligan, 1978; Federici *et al.*, 1978; Freedman, 1979). Beneking *et al.* (1978) reported an inactivating factor for tyrosine aminotransferase in rat liver that was mainly associated with the plasma membrane and with minor amounts in the mitochondria and endoplasmic reticulum. These workers proposed that inactivation probably involved formation of a disulphide bond between different cysteine residues within the enzyme or between the enzyme and an unknown particulate factor. An important feature of the above modification reactions, in common with that of the present report, is the reversibility achieved by thiol compounds (Beneking *et al.*, 1978; Federici *et al.*, 1978). These authors have speculated on the physiological significance of inactivation as part of the degradation of tyrosine aminotransferase *in vivo*. The inactivating system that we have described possesses similar properties, and in preliminary experiments we have shown that tyrosine aminotransferase is inactivated by microsomes slightly more rapidly than is glucose 6-phosphate dehydrogenase.

Several thiol-disulphide-exchange enzymes of broad specificity have been described (Freedman & Hawkins, 1977). These have been defined by a variety of assays such as re-activation of reduced proteins or cleavage of the disulphide bonds of insulin. The latter activity is often referred to as glutathione-insulin transhydrogenase (Ansorge *et al.*, 1973). Freedman & Hawkins (1977) propose a role for microsomal protein disulphide-isomerase in protein synthesis, and also argue that protein disulphide interchange may be involved in other cellular processes. To assess the relationship between inactivation factor and protein disulphide-isomerase, a co-purification of the two activities was attempted by using the method of Ansorge *et al.* (1973). However, the inactivation factor was clearly separated

during partial purification of the glutathione-insulin transhydrogenase activity from microsomes. Also, the preparation of purified inactivation factor (Francis & Ballard, 1979) used for the present experiments lacked glutathione-insulin transhydrogenase activity. The relationship of inactivation factor to these thiol-disulphide-exchange activities of cellular membranes (Freedman & Hawkins, 1977; Freedman, 1980) remains unresolved.

On the basis of the membrane inactivation-factor ability to inactivate a wide range of cytosol enzymes *in vitro* at rates roughly proportional to their degradation rates *in vivo* (Hopgood & Ballard, 1974), we postulate a general role for the factor in protein degradation. As stressed previously (Francis & Ballard, 1980), although such a relationship is very difficult to prove directly, the inactivation process studied *in vitro* certainly fulfils some of the requirements for an initial step in protein degradation. Although the reaction is initially reversible, after prolonged incubation the inactivation becomes irreversible (Fig. 3), probably indicating denaturation of the inactivated enzyme. Indeed, selective denaturation of intracellular proteins on membrane surfaces has been suggested as a means by which the proteins could be made available to lysosomal proteinases (Dean, 1975). If the inactivation factor described in the present paper is part of an intracellular degradation sequence, the different degradation rates of individual proteins would be determined by the degree of exposure of critical thiol groups in the proteins. Also, an altered degradation rate for a single protein might result from processes that could either protect or expose thiol groups. Changes in exposure might occur by ligand binding, by cofactor dissociation, as occurs with glucose 6-phosphate dehydrogenase (Bonsignore & DeFlora, 1972), or in association with enzyme phosphorylation, as occurs with tyrosine aminotransferase (Smith *et al.*, 1976).

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