Inactivation of Phosphoenolpyruvate Carboxykinase (GTP) by Liver Extracts

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1. The inactivation of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) in liver extracts was catalysed by the microsomal fraction, and led to the enzyme becoming bound to the microsomal membranes. 2. Inactivation by microsomal fraction, trypsin or heating at 48°C was accelerated by L-cystine, D-cystine and oxidized glutathione and decreased by dithiothreitol. 3. MnCl_2 and CoCl_2 protected the enzyme from inactivation by heat or microsomal fraction, but did not affect the inactivation caused by trypsin. 4. Several proteinase inhibitors had no effect on the microsomal inactivation reaction, suggesting that proteolysis was not involved. 5. It is argued that the initial step in the degradation of phosphoenolpyruvate carboxykinase (GTP) is an inactivation reaction, perhaps involving oxidized thiol compounds.

Under most physiological conditions, hepatic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is degraded with a half-life of approx. 6h (Ballard & Hopgood, 1973; Hopgood *et al.*, 1973). Attempts to use specific antibodies as probes to measure intermediates in the degradation sequence of the enzyme *in vivo* have given no indication that antibody-reactive fragments are present (Ballard *et al.*, 1974). Rather it is likely that the initial inactivation of the enzyme is the rate-limiting step in phosphoenolpyruvate carboxykinase (GTP) degradation. Although this situation may occur in intact cells, the incubation of pure radioactive enzyme with liver homogenates leads first to a loss of catalytic activity, followed by a decrease in antibody reactivity and then solubility before the detection of proteolytic-cleavage fragments (Ballard *et al.*, 1974).

Although we cannot show that inactivation reactions measured *in vitro* represent the sequence of enzyme degradation *in vivo*, an examination of the degradation rates of nine liver cytosol enzymes shows a close parallel with their rates of inactivation by liver extracts (Hopgood & Ballard, 1974). This relationship has been recorded in other studies (Bond, 1971; Salinas *et al.*, 1974; Hanson *et al.*, 1975).

In the present paper we report further investigations on the inactivation of phosphoenolpyruvate carboxykinase (GTP) *in vitro*, and in particular the intracellular location of the inactivating factor as well as the effects of bivalent cations and thiol compounds.

Materials and Methods

Chemicals

[4,5- ^3H]Leucine (sp. radioactivity 50–60 Ci/mmol) and $\text{NaH}^{14}\text{CO}_3$ were obtained from The Radio-

chemical Centre, Amersham, Bucks., U.K.; trypsin (chymotrypsin-inactivated) was from Calbiochem, Los Angeles, CA, U.S.A.; 7-amino-1-chloro-3-tosylamidobutan-2-one, phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor, lima-bean trypsin inhibitor, ox pancreas trypsin inhibitor, all enzymes and the nucleotides required for the measurement of enzyme activities were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Leupeptin, pepstatin, phosphoramidon, elastinal, bestatin and antipain were kindly given by Dr. H. Umezawa, Microbial Chemistry Research Foundation, 14-23 Kamiosaki, 3-Chome, Shinagawa-Ku, Tokyo, Japan.

Enzyme measurements

Assays for the following enzymes were carried out, at the temperature indicated, without further modifications: phosphoenolpyruvate carboxykinase (GTP), 37°C (Ballard & Hanson, 1969); glutamate dehydrogenase (EC 1.4.1.2), 25°C (Ballard, 1971); glucose 6-phosphatase (EC 3.1.3.9), 37°C (Shull *et al.*, 1956); β -glucuronidase (EC 3.2.1.31), 37°C (Bowers *et al.*, 1967); glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 25°C (Kornberg & Horecker, 1955); lactate dehydrogenase (EC 1.1.1.27), 25°C (Kornberg, 1955). A unit of enzyme is that amount of activity that catalyses either the formation of 1 μmol of product or the removal of 1 μmol of substrate/min.

Purification of phosphoenolpyruvate carboxykinase (GTP)

The enzyme used for inactivation studies had a specific activity between 3 and 5 units/mg of protein, and had been purified by the method of Ballard & Hanson (1969) up to and including the $(\text{NH}_4)_2\text{SO}_4$ extraction step.

Phosphoenolpyruvate carboxykinase (GTP),

labelled with [^3H]leucine, was prepared as described by Ballard *et al.* (1974), and had a specific activity of 15 units/mg of protein and a specific radioactivity of $0.24\ \mu\text{Ci/mg}$ of protein.

Each enzyme preparation was stored at 0°C as a suspension in $3\text{M}-(\text{NH}_4)_2\text{SO}_4$ containing 1 mM-EDTA and $1\text{ mM-dithiothreitol}$ at $\text{pH } 7$, and was stable for at least a year.

Preparation of microsomal fraction

Livers from rats that had been starved overnight were homogenized in 4 vol. of 0.25 M-sucrose at 0°C by using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 0°C and $6000g$ for 10 min, and the pellet discarded. The supernatant was centrifuged at 0°C and $300000g$ for 60 min, and the microsomal pellets were suspended in water. The suspension was freeze-dried, and the microsomal powder stored desiccated at -20°C .

Measurement of protein degradation

Radioactive cytosol proteins were prepared from livers of 3-week-old rats that had been injected with $200\ \mu\text{Ci}$ of [^3H]leucine 2 h previously. Livers were homogenized in 4 vol. of 0.25 M-sucrose , centrifuged at 0°C and $300000g$ for 60 min, and 5 ml of the clear supernatant was applied to a Sephadex G-200 column ($7\text{ cm}^2 \times 75\text{ cm}$), which was equilibrated with $10\text{ mM-potassium phosphate buffer}$, $\text{pH } 7.0$. The column was eluted with the same buffer at 0°C , and the radioactive proteins in the void volume were combined to give a volume of approx. 30 ml. This solution was dialysed against saturated $(\text{NH}_4)_2\text{SO}_4$ to precipitate the protein. The resulting suspension was kept at 0°C , and had a specific radioactivity of 6×10^4 – 10^5 d.p.m./mg of protein.

For the measurement of protein degradation, samples of the radioactive protein suspension were centrifuged at $15000g$ for 10 min, the supernatant was discarded, and the pellet dissolved in a volume of water equal to the volume of suspension taken. The solution was centrifuged at $15000g$ for 10 min to remove any insoluble material. Portions ($150\ \mu\text{l}$) of the clear solution were mixed with $150\ \mu\text{l}$ of $0.15\text{ M-potassium citrate buffer}$, $\text{pH } 4.5$, and $150\ \mu\text{l}$ of the proteinase-containing sample and incubated at 37°C with shaking at 100 oscillations/min. At 0, 2 and 4 h, $100\ \mu\text{l}$ portions were removed and mixed with $50\ \mu\text{l}$ of 30% (w/v) trichloroacetic acid. After cooling in ice and standing for at least 1 h, the trichloroacetic acid mixtures were centrifuged at $15000g$ for 10 min, and the radioactivity was measured in $50\ \mu\text{l}$ of the trichloroacetic acid-soluble solution and the complete trichloroacetic acid-insoluble pellet by using a solution composed of 0.25 ml of NCS tissue solubilizer (Amersham Searle Corp., Arlington Heights, IL, U.S.A.) and 5 ml of a scintillation fluid containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(4-

methyl-5-phenyloxazol-2-yl)benzene/litre of toluene.

Protein degradation was expressed as the percentage of total radioactivity in the trichloroacetic acid-soluble fraction after subtraction of the zero-time blank value. The endogenous proteolytic activity of the radioactive cytosol protein was less than 2% of the substrate degraded per 4 h. This method of measuring protein degradation has been adapted for use with radioactive proteins from that described by Ansorge *et al.* (1971).

Sucrose-gradient centrifugation

Freeze-dried microsomal fraction (200 mg) was homogenized in 2.5 ml of water by using a Dounce homogenizer, and 2 ml of the suspension was layered on to a linear 0.5 – 2 M-sucrose gradient (30 ml) in a polypropylene Beckman SW 25.1 rotor tube; this was centrifuged at 4°C and $60000g$ for 5 h. Sucrose (2.5 M) was pumped into the pierced bottom of the tube and 1.5 ml fractions were collected. Each fraction was treated with 10% (w/v) Triton X-100 to give a final concentration of 0.5% .

Inactivation of phosphoenolpyruvate carboxykinase (GTP)

Partially purified enzyme as a suspension in $3\text{ M}-(\text{NH}_4)_2\text{SO}_4$ was centrifuged at $15000g$ for 10 min, and the pellet dissolved in sufficient 50 mM-Tris/HCl buffer, $\text{pH } 8.0$, to give an activity of approx. 2.5 units/ml . This solution was used as a source of enzyme for methods (1)–(3) described below.

(1) *Heat inactivation.* Portions of phosphoenolpyruvate carboxykinase (GTP) solution ($20\ \mu\text{l}$) were incubated at 48°C with various additions in a total volume of $75\ \mu\text{l}$. At times from 0 to 60 min, $10\ \mu\text{l}$ samples were diluted with $50\ \mu\text{l}$ of 0.25 M-sucrose at 0°C . Enzyme activity was measured in $20\ \mu\text{l}$ of these diluted solutions.

(2) *Trypsin inactivation.* Portions of enzyme solution ($20\ \mu\text{l}$) were incubated with $6\ \mu\text{g}$ of trypsin and other additions in a total volume of $75\ \mu\text{l}$. At times up to 2 h, $10\ \mu\text{l}$ samples were diluted with $50\ \mu\text{l}$ of 0.25 M-sucrose at 0°C , and the remaining phosphoenolpyruvate carboxykinase (GTP) activity was measured.

(3) *Microsomal inactivation.* Freeze-dried microsomal fraction (10 mg) was homogenized in 1 ml of water to give a concentration of 10 mg/ml , and $20\ \mu\text{l}$ portions of this solution were incubated at 37°C with $20\ \mu\text{l}$ of phosphoenolpyruvate carboxykinase (GTP), $10\ \mu\text{l}$ of a freshly prepared solution of 10 mM-L-cystine , $\text{pH } 8.0$, and other additions in a total volume of $75\ \mu\text{l}$. At times up to 2 h, $10\ \mu\text{l}$ samples were removed, diluted with $50\ \mu\text{l}$ of 0.25 M-sucrose at 0°C , and the remaining enzyme activity was measured.

(4) *Microsomal inactivation of cytosol phosphoenolpyruvate carboxykinase (GTP).* Livers from fed rats were homogenized in 3 vol. of 0.25 M-sucrose and centrifuged at $300000g$ for 60 min. The clear super-

nant was stored at -20°C and retained phosphoenolpyruvate carboxykinase (GTP) activity for at least 2 months. Incubation mixtures contained $100\mu\text{l}$ of this cytosol solution, $50\mu\text{l}$ of freshly prepared 10mM -L-cystine, $\text{pH} 8.0$, $10\mu\text{l}$ of 0.5M -Tris/HCl buffer, $\text{pH} 8.0$, $40\mu\text{l}$ of a suspension of 10mg of freeze-dried microsomal fraction/ml of water and other additions in a total volume of $250\mu\text{l}$. Samples were taken after incubation at 37°C for up to 3h and cooled in ice for the subsequent measurement of phosphoenolpyruvate carboxykinase (GTP) activity.

In the experiments presented in Table 1 cytosol and freeze-dried microsomal fraction were replaced by $100\mu\text{l}$ of homogenate or centrifuged homogenate.

Protein measurements

Protein was determined in tissue extracts as described by Lowry *et al.* (1951), by using crystalline bovine serum albumin as a standard.

Results

Intracellular localization of the phosphoenolpyruvate carboxykinase (GTP)-inactivation reaction

Phosphoenolpyruvate carboxykinase (GTP) was inactivated when homogenates from either fed or starved rats were incubated at 37°C in the presence of L-cysteine. When L-cysteine was included in an inactivation mixture without subcellular particles, but with liver cytosol from fed rats, the enzyme was stable for up to 4h of incubation (Hopgood & Ballard, 1974). However, the replacement of L-cysteine by L-cystine in the incubation mixture accelerated the rate of inactivation, so that the enzyme was unstable even in a cytosol fraction ($100000g$ for 60min) from livers of fed rats (Table 1). Further centrifugation of the liver cytosol, so that a total field of $300000g$ was applied for 60min, produced a clear extract in which phosphoenolpyruvate carboxykinase (GTP) was virtually stable. When starved rats were used, however, there was a more rapid rate of enzyme inactivation (Table 1). Cytosol exchange studies showed that the particles in livers from starved rats were more active than particles from liver of fed rats, when incubated with cytosol from livers of fed rats. This indicated that there was no intrinsic difference in the phosphoenolpyruvate carboxykinase (GTP) from the two sources; rather the inactivating factor was present at a higher activity in livers of starved rats.

In an attempt to purify the inactivating factor, liver microsomal fraction was prepared, freeze-dried and resuspended in water. Centrifugation of this microsomal fraction on a sucrose density gradient showed that the phosphoenolpyruvate carboxykinase (GTP)-inactivating factor co-sedimented with the microsomal marker glucose 6-phosphatase (Fig. 1). Most of the β -glucuronidase activity and the small amount of mitochondrial glutamate dehydrogenase present

Table 1. Inactivation of phosphoenolpyruvate carboxykinase (GTP) by liver extracts

Livers from either fed or overnight-starved rats were homogenized in 4vol. of 0.25M -sucrose and centrifuged as indicated below. Enzyme inactivation was measured over a 90min time-period by method (4) as described in the Materials and Methods section.

Liver extract	Percentage of initial activity after incubation of liver extracts from	
	Fed rats	Starved rats
Homogenate	35	20
Supernatant, $10000g$, 10min	21	15
Supernatant, $100000g$, 15min	41	12
Supernatant, $100000g$, 60min	75	31
Supernatant, $300000g$, 60min	89	55

Fig. 1. Sucrose-gradient centrifugation of the inactivating factor

Freeze-dried microsomal fraction was suspended in water, layered on to a sucrose gradient, centrifuged and fractionated as described in the Materials and Methods section. Each fraction was analysed for protein (○), β -glucuronidase activity (▲), glucose 6-phosphatase activity (△), protein degradation in 4h with ^3H -labelled cytosol protein as substrate (●) and phosphoenolpyruvate carboxykinase (GTP) inactivated in 2h by inactivation method (4) (■). Two additional experiments gave essentially similar results.

remained at the top of the gradient. These findings, together with the localization of the proteolytic activity at the top of the gradient, suggested that both lysosomes and mitochondria were disrupted by freeze-drying, and their enzymes released.

We decided to establish whether the inactivating factor was a protein. Accordingly, it was shown that the microsomal factor was unstable to boiling, and would not pass through a dialysis membrane. Further, microsomal fraction was incubated with trypsin in the proportion of 5 parts of microsomal protein to 1 part of trypsin for 2 h. After inhibition of the trypsin with rat serum and incubation with phosphoenolpyruvate carboxykinase (GTP) by using inactivation method (4), it was shown that the inactivating capacity was diminished. This experiment could not be carried out with more trypsin because the additional serum required increased the rate of enzyme inactivation.

Several procedures were tested in order to solubilize the inactivating factor from the microsomal fraction. Treatment of microsomal fraction with 0.15M-Tris/HCl buffer, pH 8.0, 0.5M-KCl or sonication at 0°C for 1 min in a Soniprobe (Dawe Instruments, London W.3, U.K.) at maximum energy followed by centrifugation at 300000g for 60 min solubilized some microsomal protein, but not the inactivating factor. Microsomal fraction was treated with Triton X-100 to a final concentration of 0.5%, and centrifuged at 300000g for 60 min. The supernatant contained most of the inactivating factor, but subsequent chromatography of this extract on Sephadex G-100 and then on either DEAE-cellulose or CM-cellulose did not separate the factor from the bulk of microsomal protein. This was true whether or not Triton X-100 was included in the chromatography buffers. It appears therefore that the inactivating factor is

tightly incorporated in the microsomal membrane. Accordingly, the studies reported below were carried out with total microsomal fraction.

Effect of thiol compounds on enzyme inactivation

The inactivation of cytosol phosphoenolpyruvate carboxykinase (GTP) by microsomal fraction is substantially accelerated by L-cystine at 4 mM and slightly at 1 mM (Table 2). No marked effects are found when either oxidized glutathione or L-cysteine is included in the incubation medium, whereas reduced glutathione and dithiothreitol protect the enzyme from inactivation. In an attempt to determine whether the stimulatory and inhibitory effects of thiol compounds were specific to phosphoenolpyruvate carboxykinase (GTP) or due to reactions with the inactivating factor, loss of glucose 6-phosphate dehydrogenase activity was measured in the same experiments. The data in Table 2 show that the compounds had the same effectiveness as stimulants for inactivation when acting on either phosphoenolpyruvate carboxykinase (GTP) or glucose 6-phosphate dehydrogenase, thus: L-cystine > D-cystine > oxidized glutathione > no additions = L-cysteine > reduced glutathione > dithiothreitol. It might be inferred that these compounds are acting on the inactivating factor, since they had similar effects on both enzymes. However, a similar order of effectiveness for the thiol compounds was found for the heat inactivation and the trypsin inactivation of phosphoenolpyruvate carboxykinase (GTP) (Table 2).

Table 2. *Effects of thiol compounds on enzyme inactivation*

Phosphoenolpyruvate carboxykinase (GTP) inactivation by heat, trypsin or by microsomal fraction in the presence of liver cytosol was measured as described in the Materials and Methods section. The inactivation of glucose 6-phosphate dehydrogenase was also followed in the cytosol experiment.

Additions	Percentage of phosphoenolpyruvate carboxykinase (GTP) activity remaining after inactivation by			Percentage of glucose 6-phosphate dehydrogenase after 2 h microsomal inactivation
	Heat (15 min)	Trypsin (1 h)	Microsomal fraction (2 h)	
None	66	32	61	42
L-Cystine, 4 mM	10	5	10	0
1 mM	22	9	48	19
D-Cystine, 4 mM	8	8	47	10
1 mM	24	10	66	26
Oxidized glutathione, 4 mM	21	3	58	18
1 mM	53	36	61	32
Reduced glutathione, 4 mM	67	52	85	48
1 mM	73	42	86	39
L-Cysteine, 4 mM	42	48	67	33
1 mM	54	30	71	44
Dithiothreitol, 4 mM	71	84	89	78
1 mM	57	78	100	63

Fig. 2. Effect of Mn^{2+} on phosphoenolpyruvate carboxykinase (GTP) stability

Enzyme activity remaining was measured after 1 h heat inactivation (●) or 2 h inactivation by microsomal fraction by inactivation method (3) (○).

Table 3. Effects of bivalent cations on phosphoenolpyruvate carboxykinase (GTP) inactivation

Inactivation by heat, trypsin or microsomal fraction was measured by using purified enzyme. Data are expressed as the percentage of the initial activity after 1 h heat, 2 h trypsin or 2 h microsomal inactivation in the presence of the salts indicated.

Additions	Percentage of activity remaining after inactivation by		
	Heat	Trypsin	Microsomal fraction
None	15	23	15
MgCl ₂ , 2 mM	13	18	12
0.2 mM	9	20	14
MnCl ₂ , 2 mM	84	15	65
0.2 mM	81	13	54
CaCl ₂ , 2 mM	16	10	9
0.2 mM	13	16	10
CoCl ₂ , 2 mM	86	23	52
0.2 mM	90	22	50
FeCl ₂ , 2 mM	14	14	13
0.2 mM	15	11	12

Effects of cations on phosphoenolpyruvate carboxykinase (GTP) inactivation

Substrates and products of the phosphoenolpyruvate carboxykinase (GTP) reaction were tested for their effects on enzyme stabilization. We found that IDP, ITP, phosphoenolpyruvate, oxaloacetate and $KHCO_3$ had no effect on the rate of enzyme inactivation, either as individual compounds or in combination. However, $MnCl_2$ decreased phosphoenolpyruvate carboxykinase (GTP) inactivation by heat and by microsomal fraction, and was effective at a concentration of 0.1 mM (Fig. 2).

Several other bivalent cations were tested as their chloride salts for protection of the enzyme against inactivation. We found that Cu^{2+} , Zn^{2+} and Ni^{2+} inhibited phosphoenolpyruvate carboxykinase (GTP) to an extent that could not be reversed by the Mn^{2+} present in the enzyme assay. Of the other cations used, Mg^{2+} , Ca^{2+} and Fe^{2+} had no action on enzyme stability, whereas Co^{2+} protected the enzyme against microsomal inactivation at concentrations above 0.1 mM and against heat inactivation above 0.02 mM (Table 3).

The proteolytic inactivation of the enzyme was not modified by the inclusion of either $MnCl_2$ or $CoCl_2$ in the incubation mixture.

Effects of proteinase inhibitors on enzyme inactivation

Several proteinase inhibitors were tested for effects on phosphoenolpyruvate carboxykinase (GTP) inactivation by trypsin or by microsomal fraction (Table 4). Inactivation by trypsin was markedly inhibited by phenylmethanesulphonyl fluoride, 7-amino-1-chloro-3-tosylamidoheptan-2-one, rat serum, leupeptin,

Table 4. Effect of proteinase inhibitors on phosphoenolpyruvate carboxykinase (GTP) inactivation

Values are the percentage of enzyme activity remaining after incubation of purified enzyme with either trypsin or microsomal fraction for 2 h.

Additions	Percentage of activity remaining after inactivation by	
	Trypsin	Microsomal fraction
None	14	20
Phenylmethanesulphonyl fluoride, 1 mM	83	23
1-Chloro-4-phenyl-3-tosylamido-butan-2-one, 0.1 mM	15	16
7-Amino-1-chloro-3-tosylamido-heptan-2-one, 0.1 mM	71	13
Serum, 10 μ l/ml	88	18
Phosphoramidon, 50 μ g/ml	9	20
Bestatin, 50 μ g/ml	9	21
Leupeptin, 50 μ g/ml	65	18
Antipain, 50 μ g/ml	74	18
Pepstatin, 50 μ g/ml	16	20
Elastinal, 50 μ g/ml	16	16
Ox pancreas trypsin inhibitor, 100 μ g/ml	88	18
Lima-bean trypsin inhibitor, 100 μ g/ml	86	17
Soya-bean trypsin inhibitor, 100 μ g/ml	68	21

antipain and the trypsin inhibitors from ox pancreas, soya bean and lima bean, a result that is consistent with the specificity of these inhibitors on tryptic hydrolysis of other proteins. No inhibitor had any effect on inactivation of phosphoenolpyruvate carboxykinase (GTP) by microsomal fraction, although 7-amino-1-chloro-3-tosylamidoheptan-2-one produced a slight inactivation of the enzyme.

Binding of ^3H -labelled enzyme to microsomal fraction

Radioactive phosphoenolpyruvate carboxykinase (GTP) was pelleted by centrifuging the $(\text{NH}_4)_2\text{SO}_4$ suspension at 15000g for 10 min; the enzyme was dissolved in 10 mM-potassium phosphate buffer, pH 7.0, and passed through a small Sephadex G-25 column. This solution was incubated with 2 mM-L-cystine and microsomal fraction for 2 h, essentially as described for inactivation method (3), and the mixture centrifuged at 100000g for 60 min to separate microsomal particles from residual enzyme. The pellet contained approx. 40% of the radioactivity, compared with less than 5% at the beginning of the incubation. After suspension of the pellet in 10 mM-potassium phosphate buffer, pH 7.0, it was chromatographed at 0°C on a Sephadex G-200 column ($3\text{ cm}^2 \times 30\text{ cm}$) equilibrated with the same buffer (Fig. 3). Lactate dehydrogenase was added to the column as a molecular-weight marker. Essentially all the radioactivity applied to the column was eluted with the microsomal peak, with none at the elution

Fig. 3. Chromatography of microsomal-inactivated, ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) on a Sephadex G-200 column

Details of this experiment are given in the Results section. The column eluate was measured from the time at which the sample was applied. Protein was precipitated from each fraction by adding serum albumin as carrier to a concentration of 2 mg/ml, followed by treatment with trichloroacetic acid to a final concentration of 10% (w/v). The pellet was dissolved in NCS solubilizer, and the radioactivity determined (Ballard *et al.*, 1974). The arrow indicates the elution position of unmodified phosphoenolpyruvate carboxykinase (GTP). Lactate dehydrogenase (■; 60 units) was added as a molecular-weight marker. ○, Protein; ●, radioactivity.

Fig. 4. Sedimentation of microsomal-treated ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) on a sucrose density gradient

Details of the experimental procedure are given in the Results section. Measurements of protein (○) and radioactivity (●) were carried out as in the legend to Fig. 3. (a) Pellet fraction after incubation of radioactive enzyme with microsomal fraction; (b) supernatant fraction. Fractions are numbered from the top of the gradient.

volume expected for phosphoenolpyruvate carboxykinase (GTP). We interpret this experiment as showing that either the enzyme had been precipitated by the incubation conditions and, therefore, recovered in the pellet fraction, or the enzyme had been bound by the microsomal fraction.

A distinction between the two possibilities can be made because it is unlikely that precipitated enzyme would have the same equilibrium density as microsomal particles. Radioactive enzyme was pelleted, dissolved, equilibrated with phosphate buffer in a Sephadex G-25 column, incubated with microsomal fraction and L-cystine, and centrifuged as described above. The supernatant fraction and the pellet in 0.25 M-sucrose were layered on to linear sucrose gradients as described in the Materials and Methods section, and centrifuged at 50000g for 5 h. The tube was pierced and the gradient fractionated, and the amount of radioactivity and protein in each fraction was measured (Fig. 4). The supernatant radioactivity remains at the top of the sucrose gradient (Fig. 4b), and is present as undegraded phosphoenolpyruvate

carboxykinase (GTP), since little proteolysis would have occurred as a result of the incubation (Ballard *et al.*, 1974). Microsomal particles sediment in the sucrose gradient to give two main protein peaks, presumably representing smooth and rough membranes (Fig. 4a). Radioactivity is associated with both protein peaks.

In a parallel experiment, ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) was denatured by heating at 90°C for 5 min, layered on top of a sucrose gradient, centrifuged and the gradient fractionated as described above. We found that essentially all the radioactivity had sedimented through the gradient and could only be recovered by adding 1 M-NaOH to the tube after removal of the sucrose solution. It is unlikely therefore that the co-sedimentation of enzyme radioactivity with microsomal particles is related to a simple precipitation process.

Discussion

The inactivation of cytosol enzymes in liver homogenates occurs at rates approximately proportional to the degradation rates of the enzymes *in vivo* (Hopgood & Ballard, 1974). Studies with one of the enzymes tested, phosphoenolpyruvate carboxykinase (GTP), showed that the inactivating factor was associated with the microsomal fraction rather than the proteinase-containing lysosomes (Ballard *et al.*, 1974). An extension of this work in the present report indicates that the factor is heat-labile, non-diffusible and may involve a protein. Additional fractionation experiments showed that the inactivating factor could not be pelleted except by extremely high gravitational fields (Table 1), implicating small microsome-like vesicles in the reaction. We were unable to dissociate or solubilize the activity from these vesicles and concluded that the activity was an integral membrane component.

The inactivating factor is clearly separated from lysosomal cathepsins and β -glucuronidase when freeze-dried microsomal fraction is centrifuged in a sucrose gradient. Such a result implies that the factor is not intralysosomal, but it does not unequivocally eliminate a role for the lysosomal membrane. However, additional lysosomes did not accelerate the rate of enzyme inactivation in homogenates (Ballard *et al.*, 1974).

The inactivation of phosphoenolpyruvate carboxykinase (GTP) by microsomal fractions is accompanied by or followed by binding of the enzyme to membrane material. Treatment of these microsomal membranes by sonication or with various salt solutions solubilized a considerable amount of protein, but did not release the inactivated phosphoenolpyruvate carboxykinase (GTP).

Microsomal membranes have a negative charge at neutral pH (Dallner, 1974), and would not be ex-

pected to bind a negatively charged protein such as phosphoenolpyruvate carboxykinase (GTP) (Ballard & Hanson, 1969). However, microsomal membranes do bind a variety of basic proteins (Dallner, 1974), a condition that may result in local positively charged regions. It is likely that phosphoenolpyruvate carboxykinase (GTP) is inactivated before the binding reaction (Ballard *et al.*, 1974), perhaps by a thiol-exchange reaction. Should this lead to the exposure of hydrophobic sites on the enzyme molecule, then attachment to lipid-rich membranes would be feasible. Indeed it has been shown by Bohley *et al.* (1975) that proteins with short half-lives *in vivo*, such as phosphoenolpyruvate carboxykinase (GTP), have a relative abundance of hydrophobic areas.

Cystine and to a lesser extent oxidized glutathione accelerate the rate of enzyme inactivation by microsomal fraction, whereas loss of activity is retarded by dithiothreitol or reduced glutathione. These results are consistent with an inactivation exchange reaction between disulphide compounds and free thiol groups on the enzyme, perhaps catalysed by a microsomal protein disulphide isomerase or a protein disulphide reductase. However, the thiol and disulphide compounds produce similar effects on the heat inactivation of the enzyme, so that a non-enzymic process must also be involved. This concept is supported by control experiments, in which we showed that phosphoenolpyruvate carboxykinase (GTP) could be somewhat inactivated by L-cystine at 37°C in the absence of both microsomal fraction and cytosol fraction. However, L-cystine does not inactivate the enzyme in a liver cytosol fraction when the fraction is prepared from fed rats (Table 1).

Protection of the enzyme by Mn^{2+} or Co^{2+} against inactivation presumably occurs via the binding of these ions. Since both cations can function as reactants in the enzyme reaction (Snoke *et al.*, 1971), it is possible that they promote folding of the enzyme into a conformation that is less susceptible to disulphide oxidation or heat denaturation. Interestingly, trypsin inactivation was not affected by Mn^{2+} or Co^{2+} , suggesting that the proteinase-susceptible bonds remain exposed. The protection against inactivation caused by binding of Mn^{2+} may occur in the intact cell, since preliminary experiments from this laboratory show a stabilization of phosphoenolpyruvate carboxykinase (GTP) in Reuber hepatoma cells incubated with 1 mM- MnCl_2 (S. E. Knowles & F. J. Ballard, unpublished work).

There are some similarities between the results reported here with phosphoenolpyruvate carboxykinase (GTP) and inactivation studies with rabbit liver hexose diphosphatase (EC 3.1.3.11). Thus hexose diphosphatase is inactivated by oxidized glutathione, although L-cystine is not effective (Nakashima & Ogino, 1973). Further, the rate of hexose diphosphatase inactivation is extremely rapid

even at 0°C, and it can be reversed by Mn^{2+} , EDTA or ATP (Nakashima & Ogino, 1973). We have not been able to detect any reversal of phosphoenolpyruvate carboxykinase (GTP) inactivation.

Lardy and co-workers (Snoke *et al.*, 1971; Bentle & Snoke, 1974) have proposed that Fe^{2+} and not Mn^{2+} is the physiological metal cofactor for phosphoenolpyruvate carboxykinase (GTP). We note, however, that Fe^{2+} does not protect the enzyme from inactivation by either heat or microsomal fraction. This does not necessarily preclude a role for Fe^{2+} in the enzyme reactions, since Fe^{2+} may not bind directly to the enzyme but instead act through an iron-binding activator. Such an activator might participate in the stabilization of the enzyme *in vivo*, but it is unlikely that it could be involved in the present experiments.

We proposed (Ballard *et al.*, 1974) that phosphoenolpyruvate carboxykinase (GTP) degradation occurred by a sequential loss of catalytic activity, antibody reactivity and solubility before any proteolytic cleavage. The data in the present paper are in general agreement with such a degradation sequence, and strongly support the proposal that proteolytic reactions are not involved in the early stages of inactivation. However, the lack of solubility after inactivation of the enzyme is clearly a binding of the protein to microsomal membranes rather than a simple precipitation. The subsequent proteolytic degradation of inactivated phosphoenolpyruvate carboxykinase (GTP) *in vivo* might occur directly by microsomal proteinases or perhaps after fusion of membranes and attached inactivated enzyme with lysosomes. In the latter case degradation could take place via the cathepsins within these organelles.

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