Effect of physiological reducing compounds on the inactivation of tyrosine aminotransferase from guinea-pig liver*

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1. Tyrosine aminotransferase from guinea-pig liver is inactivated at neutral pH by a factor localized in the microsomal fraction. The inactivation, independent of exogenous L-cysteine, is rapidly reversed by addition of dithiothreitol. 2. The effects of physiological reducing agents on the enzyme inactivation were investigated. L-Cysteine and L-cysteamine enhance the inactivation rate of the enzyme in the presence of microsomal membranes, and also they are able to bring about the loss in enzyme activity independently of microsomal action. Reduced glutathione, at physiological concentration, and NADPH decrease the inactivation rate. Other physiological reducing compounds, as well as oxidized glutathione and NADP⁺, are without effect. 3. Neither reduced glutathione nor NADPH, unlike dithiothreitol and mercaptoethanol, is able to restore the activity of partially inactivated tyrosine aminotransferase. 4. It is proposed that the intracellular concentration of reduced glutathione might modulate the rate of inactivation of the enzyme *in vivo*.

The short half-life of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) *in vivo* makes this enzyme a convenient model for studies of regulatory processes of protein degradation in mammalian cells.

Although the overall process of intracellular inactivation and degradation of tyrosine aminotransferase has been regarded as a multi-step phenomenon (Grossman & Bocktor, 1972), the exact sequence of events and the control mechanisms are not yet elucidated.

Several studies (Ohsalo, 1977; Beneking *et al.*, 1978; Federici *et al.*, 1978; Buckley & Milligan, 1978) have pointed out that one or more thiol group(s) of the enzyme, essential for catalytic activity, easily undergo chemical modification. L-Cysteine, spontaneously converted into L-cystine, can form a mixed disulphide with purified enzyme, leading to a reversible loss of activity (Federici *et al.*, 1978). In the cell-free system from rat liver the inactivation of tyrosine aminotransferase by L-cysteine seems to proceed through an oxidation of

thiol group(s) of the enzyme, catalysed by a factor predominantly localized in the cytoplasmic membrane (Beneking *et al.*, 1978).

In spite of the significance of these oxidative reactions, which may reflect intracellular events, it is not known whether such a process is regulated by modulating factors. Therefore we have examined the effects of major physiological reducing agents on the inactivation of tyrosine aminotransferase *in vitro*.

Experimental

Animals

Male albino guinea pigs (body wt. 400–500 g), obtained from Istituto Zooprofilattico of Teramo (Italy) and maintained on regular laboratory food and water *ad libitum*, were used in all experiments.

Preparation of liver extracts

Guinea-pig livers were washed in cold 0.9% NaCl, weighed, minced and homogenized in a glass/Teflon tissue grinder in 8 vol. (v/w) of Krebs–Ringer phosphate buffer (De Luca & Cohen, 1964), pH 7.4, or in 20 mM-potassium phosphate buffer, pH 7.4, containing 0.25 M-sucrose. Homogenization was accomplished with ten up-and-down strokes in crushed ice.

Abbreviations used: GSH and GSSG, reduced and oxidized glutathione respectively.

* Dedicated to Professor Alessandro Rossi Fanelli for his 75th birthday.

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Subcellular fractionation

Subcellular crude fractions were separated by differential centrifugation by a typical procedure essentially derived from that of de Duve *et al.* (1955). The liver, homogenized in Krebs–Ringer phosphate buffer, pH 7.4, containing 0.25 M-sucrose, was centrifuged at 600 *g* for 10 min to sediment nuclei and debris. The supernatant was centrifuged at 15 500 *g* for 20 min to remove mitochondria and lysosomes. The microsomal fraction was finally pelleted by centrifuging the previous supernatant at 100 000 *g* for 60 min. All operations were performed at 4°C in a Beckman J-21C centrifuge or in a Beckman (Spinco) ultracentrifuge with a 50 Ti rotor.

Preparation of the standard system for the inactivation measurements

Unless otherwise indicated, to measure the inactivation of tyrosine aminotransferase the homogenate, prepared in Krebs–Ringer phosphate buffer, pH 7.4, was centrifuged at 15 500 *g* for 20 min. The supernatant was incubated with gentle shaking at 37°C. Samples were withdrawn at intervals and assayed for tyrosine aminotransferase activity.

Assay of tyrosine aminotransferase activity

Tyrosine aminotransferase activity was assayed by the method of Diamondstone (1966), with slight modifications. Calculated concentrations of reagents in the final (2.0 ml) assay mixture were: potassium phosphate buffer, 90 mM; EDTA, 1 mM; L-tyrosine, 3 mM; 2-oxoglutarate, 7 mM; pyridoxal 5'-phosphate, 0.05 mM; sodium diethyldithiocarbamate, 3.5 mM. At 37°C the pH of the enzyme assay mixture including homogenate was 7.8. The reaction, started by addition of 2-oxoglutarate, was stopped after 30 min incubation at 37°C by the addition of 10 M-KOH to give a final concentration of 0.5 M. The absorbance at 331 nm ($\epsilon = 19\,900\text{ M}^{-1}\cdot\text{cm}^{-1}$) was read against a blank in a Gilford spectrophotometer. The enzyme activity, obtained by the addition of 0.025 ml of tissue extract, was expressed as μmol of product formed/min.

Measurements of glutathione and L-cysteine

Fresh hepatic tissues were homogenized, in a glass/Teflon grinder, in ice-cold 6% (w/v) metaphosphoric acid to give a 10% (w/v) tissue dispersion. Precipitated proteins were centrifuged off, and the samples of supernatant were adjusted to pH 8 with KOH. GSH was immediately assayed by the method of Ellman (1959). GSSG measurement was performed as described by Eriksson (1966).

L-Cysteine was determined by analysis with an amino acid analyser. Tissue extraction was made as described for glutathione measurements with the substitution of 5% (w/v) trichloroacetic acid for

methaphosphoric acid. A suitable volume of clarified supernatant was extracted twice with diethyl ether, dried *in vacuo*, resuspended with 0.1 M-HCl and then analysed with an Aminolyzer (Optica, Milan, Italy), with norleucine as internal standard.

Replication of experiments

The data reported are mean values from at least three experiments, each varying within 10% of the mean.

Chemicals

L-Cysteine, L-cysteamine, 2-oxoglutaric acid, sodium diethyldithiocarbamate, L-tyrosine, ascorbic acid, 2-mercaptoethanol and dithiothreitol were all obtained from Merck (Darmstadt, Germany). Pyridoxal 5'-phosphate, NADPH, NADP⁺, NADH, NAD⁺, GSH and GSSG were purchased from Biochemia Boehringer (Mannheim, Germany). Sephadex G-25 was supplied by Pharmacia (Uppsala, Sweden). Other products were of the best available grade.

Results

When homogenates from guinea-pig liver are incubated at 37°C, tyrosine aminotransferase is progressively inactivated (Fig. 1). The inactivation

Fig. 1. Inactivation of tyrosine aminotransferase in neutral homogenates

Experimental conditions of tissue homogenization, subcellular fractionation and enzyme activity measurements were as described in the Experimental section. ●, Crude homogenate; ■, 600 *g* supernatant; □, 15 500 *g* supernatant; ▲, 100 000 *g* supernatant.

follows first-order kinetics with respect to time. The half-life of the enzyme (about 4 h) is not significantly modified by the removal of nuclei, mitochondria and lysosomes, but is greatly increased by the removal of crude microsomal fraction (Fig. 1).

Since the inactivating factor appeared to be localized in microsomal particles, in all further inactivation experiments nuclei, mitochondria and lysosomes were eliminated from the incubation medium to minimize possible interfering effects caused by the release of cathepsins and converting factor from lysosomes (Auricchio *et al.*, 1972; Boctor & Grossman, 1978; Gohda & Pitot, 1980).

The effects of physiologically important reducing agents on tyrosine aminotransferase inactivation are shown in Figs. 2 and 3. Among the compounds tested GSH and NADPH decrease the inactivation rate, L-cysteine and L-cysteamine increase it, and NADH and ascorbic acid are without effect (results not shown). The presence of 0.25 M-sucrose in the homogenizing Krebs-Ringer phosphate buffer did not modify the experimental results.

The action of L-cysteine was tested at different concentrations of the amino acid. At 5 mM L-cysteine enhances the inactivation of the enzyme in the presence of microsomal fraction, and also brings about the loss in enzyme activity even in the absence of microsomal particles. When L-cysteine, which is present in guinea-pig liver at a concentration

between 40 and 70 nmol/g fresh wt. of tissue, is added at physiological amount (0.05 mM), negligible effect is observed (Figs. 2a and 2c).

The presence of 2–8 mM-GSH in the incubation medium increases stabilization of the enzyme (Figs 2b and 3). The concentration of GSH in guinea-pig hepatic tissue, with a mean value of 5.2 mM (s.d. ± 0.7) (five observations), is in this range. GSH added to incubation mixtures undergoes rapid oxidation, so that more than 60% and 25% of initial concentrations at 2 mM and 8 mM respectively may be found in the oxidized form after 4 h incubation.

At 4 mM NADPH is also effective in counteracting enzyme inactivation. The protective action is further stimulated by the simultaneous addition of GSH (Fig. 3).

At 5 mM GSSH, as well as 4 mM-NADP⁺ 4 mM-NADH, 4 mM-NAD⁺ or 10 mM-ascorbic acid does not affect the inactivation rate of tyrosine aminotransferase (results not shown).

In view of the rapid oxidation of GSH, an experiment was performed to verify whether the protective action of NADPH might be mediated by GSH continuously regenerated by cytosolic NADPH-dependent glutathione reductase. Therefore we attempted to eliminate endogenous free GSH and GSSG from the incubation mixture. Cytosolic and microsomal fractions were obtained as described in the Experimental section from a single

Fig. 2. Effects of physiological reducing compounds on tyrosine aminotransferase activity

Tissue homogenization was performed as described in the Experimental section in Krebs-Ringer phosphate buffer, pH 7.4. The homogenate was centrifuged at 15 500 g for 20 min (a and b) or at 100 000 g for 60 min (c), and the supernatants were incubated with shaking at 37°C. Each compound was dissolved in the homogenizing medium and neutralized, then added to the centrifuged homogenate to give the final concentration values indicated. (a) ●, No addition; ▲, 0.05 mM-L-cysteine; ■, 5 mM-L-cysteine; □, 5 mM-L-cysteamine. (b) ●, No addition; ■, 2 mM-GSH; ▲, 4 mM-GSH; □, 8 mM-GSH. (c) ●, No addition; ▲, 0.05 mM-L-cysteine; ■, 5 mM-L-cysteine; □, 5 mM-L-cysteamine.

Fig. 3. Effect of NADPH at increasing concentration of GSH

Tissue homogenization and centrifugation was performed as described in the legend to Fig. 2. The supernatant was treated with increasing concentrations of GSH in the absence (●) or in the presence of 4 mM-NADPH (□). After 4 h incubation samples were withdrawn and assayed for enzyme activity.

homogenate (4 ml) prepared with Krebs–Ringer phosphate buffer. Cytosol (3 ml) was filtered through a Sephadex G-25 column (1.5 cm × 25 cm), eluted with the homogenizing medium and fractionated into 0.6 ml samples. The elution profile at 280 nm shows two major peaks. Tyrosine aminotransferase and glutathione reductase activities were found in the first peak, eluted with the void volume, and free GSH and GSSG were localized in the second peak, containing also endogenous cysteine. The tubes with enzyme activity were collected and pooled. This preparation was used to resuspend the microsomal pellet with the aid of two or three strokes of a Potter homogenizer. A small volume of the suspension was directly utilized for control samples. The remaining part was divided into two fractions. To both was added 4 mM-NADPH, and to one of them was added 2 mM-GSH also. After 4 h incubation tyrosine aminotransferase, in the sample containing NADPH alone, retains 62% of initial activity compared with 48% for a blank control (no addition). The fraction provided with NADPH and GSH maintains more than 75% of the enzyme activity, compared with 55% for the control sample to which only GSH (2 mM) had been added.

In view of the possibility that tyrosine aminotransferase inactivation may be reversed by dithiothreitol (Beneking *et al.*, 1978; Di Cola & Federici, 1982) and 2-mercaptoethanol (Federici *et*

Fig. 4. Effects of reducing agents on the re-activation of inactivated tyrosine aminotransferase

The homogenate was centrifuged at 15 500g for 20 min. The supernatant was initially incubated at 37°C in the absence (●—●) or in the presence of 5 mM-L-cysteine (●—●). After 3 h incubation the samples were divided into several fractions and reducing compounds were added: ■, 5 mM-dithiothreitol; ▲, 5 mM-2-mercaptoethanol; ○, 5 mM-GSH; □, 5 mM-NADPH.

et al., 1978), we checked the ability of physiologically active reducing agents to restore the activity of partially inactivated tyrosine aminotransferase. Although the addition of dithiothreitol or 2-mercaptoethanol restores enzyme activity almost completely (Fig. 4), none of the physiological reducing compounds shows a similar effect. It is noteworthy that, when the inactivation of the enzyme is performed in the presence of 5 mM-L-cysteine, the efficacy of dithiothreitol or 2-mercaptoethanol is drastically diminished.

Discussion

Tyrosine aminotransferase from guinea-pig liver is inactivated in a cell-free system at neutral pH by a factor localized in the microsomal fraction.

In contrast with previous reports for rat liver (Auricchio *et al.*, 1972; Beneking *et al.*, 1978; Buckley & Milligan, 1978), the inactivation of tyrosine aminotransferase from guinea-pig liver occurs without the addition of L-cysteine. The inactivation does not seem to be mediated by endogenous free cysteine, since the concentration of this amino acid in hepatic tissue is very low, and even its removal by gel filtration does not affect the inactivation by microsomal fraction.

However, some caution is required before it is

assumed that the inactivating systems of guinea-pig liver and rat liver are really different. In fact, the authors who worked with rat liver (Auricchio *et al.*, 1972; Beneking *et al.*, 1978; Buckley & Milligan, 1978) tested tyrosine aminotransferase by the method of Granner & Tomkins (1970), which requires the presence of dithiothreitol in the assay mixture, whereas we have used the method of Diamondstone (1966), which does not require such a reducing agent. Since dithiothreitol almost totally reverses tyrosine aminotransferase inactivation in the absence of L-cysteine, but not in its presence, it is highly probable that the presence of dithiothreitol in the assay mixture could re-activate the enzyme, thereby concealing a cysteine-independent inactivating process.

Although L-cysteine, as previously reported (Federici *et al.*, 1978) and confirmed in the present work, is able to inactivate tyrosine aminotransferase *in vitro*, this amino acid is present in hepatic tissue of the guinea pig at a concentration too low to exert a significant role in the removal of the enzyme activity *in vivo*.

It has been shown (Beneking *et al.*, 1978) that GSH, unlike L-cysteine, is unable to inactivate tyrosine aminotransferase. Our results show that GSH, not only does not inactivate the enzyme, but even protects it from the inactivating system. Since GSH is effective *in vitro* in a physiological concentration range, it is possible that such a compound could act *in vivo* as a modulating factor for the process of the inactivation of tyrosine aminotransferase.

The protective action of NADPH is specific, since its related analogues (NADP⁺, NADH, NAD⁺) are all totally ineffective. The exact mechanism of NADPH protection remains to be elucidated. The gel-filtration experiment shows that the removal of endogenous GSH and GSSG diminishes the NADPH protective action, but does not totally abolish it. Therefore NADPH, although it might regenerate GSH through endogenous glutathione reductase, appears to be able to stabilize the enzyme from inactivation by a different and unknown mechanism. It has been reported (McCay *et al.*, 1981) that microsomal fraction in the presence of NADPH can produce lipid peroxidation of mem-

branes. It is possible that such an event might destroy the native environment of the microsomal inactivating factor, lowering its activity.

Although more direct proof is required, our present results fit very well the scheme previously proposed (Francis & Ballard, 1980) for the inactivation of glucose 6-phosphate dehydrogenase and some other cytoplasmic enzymes. Accordingly, tyrosine aminotransferase appears to be inactivated *in vitro* by a disulphide-thiol exchange catalysed by a microsomal membranous factor. GSH may play a significant role in the protection of enzyme, competing against the inactivating system.

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