Participation of Cysteine and Cystine in Inactivation of Tyrosine Aminotransferase in Rat Liver Homogenates

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1. Inactivation of tyrosine aminotransferase was studied in rat liver homogenates. Under an O₂ atmosphere with cysteine added, inactivation was rapid after a lag period of approx. 1 h, whereas a N₂ atmosphere extended the lag period to approx. 3 h. 2. Replacement of cysteine with cystine resulted in rapid inactivation both aerobically and anaerobically. 3. Removal of the particulate fraction by centrifuging rat liver homogenates at 13000g for 9 min resulted in an aerobic lag period of 0.5 h in the presence of cystine and approx. 3 h in the presence of cysteine. 4. It is proposed that the stimulatory effect of cysteine on tyrosine aminotransferase inactivation occurs largely as a result of oxidation to cystine, which appears to be a more directly effective agent.

The inactivation of tyrosine aminotransferase (L-tyrosine-2-oxoglutarate aminotransferase, EC 2.6.1.5) in cell-free systems at neutral pH has been investigated in several laboratories (Auricchio et al., 1972; Reynolds & Thompson, 1974; Hannah & Sahib, 1975; Seubert & Hamm, 1975), and may reflect reactions in the intracellular turnover of this enzyme. A common factor in these systems in vitro has been the addition of cysteine.

Auricchio et al. (1972) have reported that tyrosine aminotransferase was rapidly inactivated in crude rat liver homogenates after the addition of 10 mm-cysteine. Reynolds & Thompson (1974) obtained similar results, but observed that enzyme inactivation was preceded by a lag period of about 1 h during which the enzyme activity was stable. Seubert & Hamm (1975) have reported that ATP inhibits the rapid inactivation of tyrosine aminotransferase in the presence of 5 mm-cysteine and Hannah & Sahib (1975) observed rapid inactivation of purified tyrosine aminotransferase in the presence of a crude lysosome fraction and 10 mm-cysteine. The purpose of the present study was to investigate further the role of cysteine in the inactivation of tyrosine aminotransferase in vitro.

Experimental

Chemicals

Bovine serum albumin (fraction V), L-cysteine, L-cystidine, 2-oxoglutaric acid, pyridoxal 5'-phosphate, sodium diethyldithiocarbamate, triamcinolone (9α-fluoro-16α-hydroxyprednisolone diacetate), Triton X-100 and L-tyrosine were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Animals and preparation of liver homogenates

Male Sprague–Dawley rats weighing between 80 and 145 g were injected intraperitoneally with 1–2 ml of a suspension of triamcinolone in 0.15m-NaCl to result in a dose of 15 or 30 mg/100 g body wt. at 10, 11 or 16 h before killing. In preliminary experiments it was found that varying the time between injection and killing from 11 to 22 h had no effect on the inactivation of tyrosine aminotransferase in liver homogenates (Buckley, 1975). Immediately after killing by decapitation, the livers were removed, washed in cold tap water and immersed in 0.15m-NaCl at 0–2°C. Each liver was weighed, minced and homogenized with a glass/Teflon tissue grinder in 10 vol. (v/w) of Krebs–Ringer phosphate buffer (De Luca & Cohen, 1964) or Tris/citrate buffer (prepared by mixing 0.31 m-Tris and 0.31 m-citric acid to obtain the desired pH) plus any additions. The Teflon pestle of the tissue grinder was machined such that it was 0.21 mm less in diameter than a normal-fitting pestle. This was assumed to be similar to the ‘very loose fitting’ pestle described by Auricchio et al. (1972). The Teflon pestle was driven by an electric hand drill for which the rotational velocity was controlled at approx. 700rev./min. Homogenization was accomplished with ten up/down strokes. The homogenates were centrifuged in a fixed-angle rotor (International Equipment Co., B-20 refrigerated centrifuge, rotor no. 870, r(max). 7.25 cm). Homogenate preparation, including centrifugation, was carried out at 0–5°C. Centrifugation was completed approx. 40 min after killing a rat. At this time the homogenate was rapidly warmed to 37°C.
Incubations

After warming to 37°C, the pH of homogenates was measured with a combination electrode; the pH of Tris/citrate-buffered homogenates was adjusted, if necessary, to 7.5 unless specified otherwise by using 0.31 M-Tris or 0.31 M-citric acid; the pH of homogenates prepared with Krebs–Ringer phosphate buffer (7.2–7.3 at 37°C) was not adjusted. The homogenates were incubated at 37°C with continuous stirring or shaking. Unless described differently, the atmosphere over the homogenates was controlled by admitting water-saturated N₂ or O₂ (37°C) into the incubation vessel throughout the incubations. For experiments specifying an air atmosphere, the inlet and outlet for gas exchange were left open to the air. During incubation the homogenates were continuously filtered through a milk filter (gauze-faced absorbent cotton) in a recirculating closed system to remove precipitated proteins. Homogenate was continuously pumped from the incubation vessel for immediate automated assay of tyrosine aminotransferase activity. About 6–10 min lapse between warming the homogenate and the beginning of the continuous enzyme assay. Incubation periods varied from 2.5 to 6 h, with rapid enzyme inactivation resulting in earlier termination of the incubation.

Tyrosine aminotransferase assay

Tyrosine aminotransferase was assayed by the method of Granner & Tomkins (1970), with modifications including the addition of sodium diethylthiocarbamate as described by Diamondstone (1966) and substitution of Tris buffer for potassium phosphate buffer. The assay method was also automated (Buckley, 1975), which required several other minor changes, including the addition of 0.03% (v/v) Triton X-100 to prevent precipitation of material on inside walls of tubing and flow cells.

AutoAnalyzer (Technicon Instruments Corp. Tarrytown, NY, U.S.A.) components were used for the automated tyrosine aminotransferase assay. Enzyme activity was measured in a continuous-flow system by the addition of reagents to the homogenate stream pumped at a constant rate of 0.05 ml/min from the homogenate incubation vessel. Calculated concentrations of reagents and homogenate in the assay mixture were: homogenate, 6.0 μl/ml; Tris, 84 mM; EDTA, 1 mM; bovine serum albumin (fraction V), 0.18 mg/ml; L-tyrosine, 6.7 mM; 2-oxoglutaric acid, 13 mM; pyridoxal 5'-phosphate, 0.13 mM; dithiothreitol, 0.82 mM; sodium diethylthiocarbamate, 3.4 mM; Triton X-100, 0.03% (v/v). At 37°C the pH of the enzyme assay mixture including homogenate was 7.6. After 30 min incubation at 37°C, enzyme activity was stopped by the addition of 6 M-KOH to the reactant stream to give a concentration of 0.64 M. As the alkaline reaction mixture passed through a flow cell (10 mm path length) the A₃₃₁ was recorded at 30 s intervals by using a recording spectrophotometer (model 2000, Gilford Instrument Laboratories, Oberlin, OH, U.S.A.). A blank was run simultaneously by splitting the assay stream and adding KOH before incubation to one half of the flow. The blank absorbance was either recorded or compensated for electronically. Thus the relative changes in enzyme activity were continuously recorded for each liver homogenate. Since dilution values, flow rates and incubation times were not precisely determined in the automated enzyme-analysis procedure, enzyme activity is not presented as specific activity. However, one A₃₃₁ unit was equal to approx. 26 nkat/ml of homogenate. Data points have been taken at 15 min intervals from the recorded curves and plotted either directly as absorbance or as percentage of maximum enzyme activity.

Results

Initial experiments were carried out with livers homogenized in Krebs–Ringer phosphate buffer. When homogenates were incubated under an air atmosphere tyrosine aminotransferase was slowly inactivated, with 86% of the maximum activity remaining after 4 h (Fig. 1a). Under an O₂ atmosphere the rate of inactivation was greater, particularly during the first hour of incubation (Fig. 1b). After 4 h of incubation twice as much enzyme activity was lost under O₂ as under air, and approximately three times that under a N₂ atmosphere (Fig. 1c).

Because of the reported competition between phosphate and pyridoxal 5'-phosphate for binding to tyrosine aminotransferase (Hayashi et al., 1967), Tris/citrate buffer was substituted for Krebs–Ringer phosphate in subsequent experiments. Buffers with concentrations of Tris plus citric acid less than 0.31 M did not maintain a constant pH throughout the incubation. In the presence of cysteine, the rate of enzyme inactivation, estimated as the maximum negative slope of the inactivation curve, was considerably higher for the range of pH from 7.5 to 9.0 than at pH 6.5 or 7.0 (Fig. 2). A lag period was observed from pH 6.5 to 7.5; the lag was about 0.75 h at pH 6.5 and approx. 1 h at pH 7.5. At pH 8.0, 8.5 and 9.0 initial enzyme activity was lower than when the pH was closer to neutrality. Subsequent investigations were carried out at pH 7.5.

There was no inactivation of tyrosine aminotransferase in Tris/citrate-buffered homogenates incubated under O₂ with cysteine omitted (Fig. 3a). Addition of 9.1 mM-cysteine resulted in rapid tyrosine aminotransferase inactivation after a 1 h lag period (Fig. 3b). When the supernatant from centrifuging the homogenate at 13000g for 9 min was incubated rather than the whole homogenate, there was a decrease in the
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Fig. 1. Inactivation of tyrosine aminotransferase in rat liver homogenates under (a) air, (b) \( \text{O}_2 \) or (c) \( \text{N}_2 \) atmosphere

Whole livers were homogenized in 10vol. (v/w) of Krebs–Ringer phosphate buffer and centrifuged at 750g for 10 min, except for (c), which was centrifuged at 84g for 5 min. The pH of the homogenates at 37°C was 7.2–7.3. Relative changes in enzyme activity were measured in the homogenates incubated at 37°C. Zero time on the graphs represents the beginning of the continuous enzyme assay, which was 6–8 min after warming the homogenate from 0–5°C to 37°C as described in the text. ●, Means of values taken from two enzyme-activity curves recorded by continuous automated analysis as described in the text. Where the range of the two values is larger than the diameter of the ● symbols, the range is indicated by a vertical bar. ■, Single points taken from one or two curves where duplicates are not available owing to missing values. The line in (b) has been interpolated where only single points are shown. In (c) the incubation vessel was sealed after flushing with \( \text{N}_2 \). An attached gas bag permitted volume changes at atmospheric pressure. All points in (c) are taken from a single curve.

Discussion

Auricchio et al. (1972) and Reynolds & Thompson (1974) reported a stimulation of tyrosine aminotransferase inactivation by cysteine in rat liver homogenates. In their experiments, livers were homogenized in a modified Krebs–Ringer phosphate buffer, and inactivation of tyrosine aminotransferase was relatively slow in the absence of added cysteine. We have also found that inactivation in rat liver homogenates prepared with Krebs–Ringer phosphate buffer without added cysteine is relatively slow, but is stimulated by the availability of \( \text{O}_2 \) (Fig. 1). Apparently, Auricchio et al. (1972) and Reynolds & Thompson (1974) did not supply \( \text{O}_2 \) to homogenates. In Tris/citrate-buffered homogenates without added cysteine or cystine, tyrosine aminotransferase was stable under an \( \text{O}_2 \) atmosphere, but showed rapid aerobic inactivation after a 1 h lag period in the presence of 9.1 mM-cysteine (Figs. 3a and 3b). Reynolds & Thompson (1974) reported the existence of a lag period before the inactivation of tyrosine aminotransferase in the presence of cysteine under an atmosphere of air.

The decrease in the rate of tyrosine aminotransferase inactivation and extension of the lag period in the presence of cysteine and \( \text{O}_2 \) as a result of centri-
fuging the homogenate at 13000 g (Fig. 3c) rather than 270 g differed from the report of Auricchio et al. (1972), who observed complete inhibition of inactivation after centrifuging homogenates at 13000 g. Auricchio et al. (1972) suggested that lysosomes, which should have been removed by their centrifuge treatment, were the site of tyrosine aminotransferase inactivation and that the stimulatory effect of cysteine was to activate cathepsins B and B1. They (Auricchio et al., 1972) demonstrated that cathepsins B and B1 inactivated tyrosine aminotransferase at pH 5. However, it should be noted that cytochrome c plus cytochrome c oxidase (EC 1.9.3.1) (Keilin, 1930), as well as whole rat liver mitochondria (Wainer, 1967), catalyse the oxidation of cysteine to cystine; doubtless the quantities of these catalysts would have been decreased in the supernatant after centrifuging at 13000 g as compared with the whole homogenates.

We considered that the present observations of the marked delays of inactivation in the presence of cysteine caused by omission of O2 or of the particulate material that sedimented at 13000 g may indicate that oxidation of cysteine to cystine was a necessary occurrence for early cysteine-mediated inactivation. This interpretation was supported by the finding that cysteine would replace cysteine in the inactivation of tyrosine aminotransferase in the present study. Indeed, in the presence of cysteine, inactivation proceeded readily in N2 as well as O2 and with supernatants from homogenates that had been centrifuged at 13000 g for 9 min. Thus the stimulatory effect of cysteine on tyrosine aminotransferase inactivation may entail its oxidation to cystine, which in turn appears to have a more direct role in the inactivation process.

![Fig. 3. Inactivation of tyrosine aminotransferase in rat liver homogenates supplemented with 9.1 mM-cysteine](image)

Whole livers were homogenized in 10 vol. (v/w) of Tris/citrate buffer. The pH of the homogenates at 37°C was adjusted to 7.5 if necessary. Homogenates were incubated under O2 at 37°C and the relative changes in tyrosine aminotransferase activity were measured. (a) No cysteine; homogenates were centrifuged at 270 g for 10 min before incubation. (b) With 9.1 mM-cysteine: homogenates were centrifuged as in (a). (c) With 9.1 mM-cysteine: the homogenate was centrifuged at 13000 g for 9 min. For (a), (b) and (c), means of values taken from two enzyme-activity curves are as described in Fig. 1.

![Fig. 4. Inactivation of tyrosine aminotransferase in rat liver homogenates supplemented with 4.5 mM-cystine](image)

(a) Homogenates centrifuged at 270 g for 10 min; means of four enzyme-activity curves. (b) Homogenates centrifuged at 13000 g for 9 min; means of two enzyme-activity curves. Ranges larger than the diameter of the ● symbols are indicated by vertical bars. Other conditions are as described in Fig. 3.
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That centrifuging at 13000g did not completely prevent inactivation in the presence of cysteine and O₂ might be expected, since traces of various metals have long been known to catalyse the oxidation of cysteine (Keilin, 1930). Thus centrifuging would not have brought about sedimentation of all of the capacity for catalysis of oxidation of cysteine to cystine. The inactivation of tyrosine aminotransferase in the presence of cysteine disagrees with the observation of Reynolds & Thompson (1974), who reported that cystine, as opposed to cysteine, had no effect on enzyme activity. Since we observed a different response with Krebs–Ringer phosphate buffer compared with Tris/citrate buffer (Figs. 1b and 3a) while other conditions remained unchanged, this discrepancy may be due to homogenization of livers with Krebs–Ringer phosphate buffer in the experiments of Reynolds & Thompson (1974), as opposed to homogenization with Tris/citrate buffer in most of our inactivation studies.

The suggestion that cysteine may be oxidized to cystine before enzyme inactivation is apparently inconsistent with two observations. The short lag period preceding aerobic inactivation in the presence of cystine after centrifuging at 13000g for 9 min (Fig. 4b) is not explained, nor is the eventual inactivation of tyrosine aminotransferase in the presence of cysteine under N₂ (Fig. 5b). To investigate the first anomalous observation, 18 mM-Na₄P₂O₇ plus 0.18 mM-CuSO₄, which catalyse the oxidation of cysteine to cystine (Keilin, 1930), were included in supernatants of homogenates that contained cystine and had been centrifuged at 13000g for 9 min. The purpose of centrifuging was to remove subcellular particles that would catalyse oxidation of endogenous cysteine. The addition of Na₄P₂O₇ plus CuSO₄ resulted in elimination of the lag period and allowed rapid aerobic inactivation to proceed immediately. Since we had noted previously that the lag period preceding inactivation was accentuated in the presence of cysteine compared with cystine, it appeared then that the aerobic lag period in the presence of cystine (Fig. 4b) may have been due to endogenous cysteine in the homogenates, and that endogenous cysteine may have had a protective effect on the enzyme. This suggestion is only tentative, as further studies were not carried out to determine the possible effects of Na₄P₂O₇ plus CuSO₄ other than oxidation of cysteine, although Na₄P₂O₇ alone with cystine did not influence the lag period or rate of inactivation.

The second anomalous observation, delayed anaerobic inactivation, was not investigated further. The delayed anaerobic inactivation of tyrosine aminotransferase (Fig. 5b) might have been a component of total inactivation that entailed direct involvement of cysteine, as suggested by Auricchio et al. (1972).

A variety of enzymes are inactivated by cystine or other disulphides (Pihl & Sanner, 1961; Scheuch & Rapoport, 1962; Hill & Kanarek, 1964; Scheuch et al., 1964; Bradshaw et al., 1967; Ballard & Hopgood, 1976), at least some, if not all, by the formation of a mixed disulphide between the low-molecular-weight disulphide and the enzyme molecule (Pihl & Sanner, 1961; Hill & Kanarek, 1964; Bradshaw et al., 1967). Thus, although conclusive evidence is presently lacking, it is certainly possible that the inactivation of tyrosine aminotransferase in the presence of cystine in the experiments reported in the present paper may also have been due to mixed-disulphide formation. This hypothesis is strengthened by evidence that a reactive thiol group is in or near the active site of tyrosine aminotransferase (Kenney, 1959; Holten et al., 1967). Since oxidation of cysteine to cystine would be expected under aerobic conditions, the stimulation of tyrosine aminotransferase inactivation measured aerobically on the addition of cysteine to homogenates may also be due to mixed-disulphide formation.

At present, the significance in vivo of the inactivation of tyrosine aminotransferase observed in this study has not been established. Considering the possibility that the inactivation of tyrosine aminotransferase may have been due to mixed-disulphide formation, it is noteworthy that mixed disulphides of proteins with low-molecular-weight thiol compounds, including glutathione and cysteine, have been identi-
fied in a variety of tissues (Modig, 1968; Harrap et al., 1973).

While the present work was being prepared for publication, Beneking et al. (1978) reported that a factor associated mainly with plasma membranes inactivated purified tyrosine aminotransferase in the presence of cysteine. The rate of inactivation varied between different forms of the enzyme, and it was suggested (Beneking et al., 1978) that the variation was due to the time required for conversion to the most readily inactivated form. The existence of multiple forms of tyrosine aminotransferase has been demonstrated by several laboratories, and Rodriguez & Pitot (1976) have found that the multiple forms possess different numbers of thiol groups. A nondiffusible component associated with the lysosomal and mitochondrial fraction of rat liver was shown to catalyse the conversion from the form of the enzyme with more thiol groups to the forms with fewer thiol groups (Rodriguez & Pitot, 1976). These recent developments indicate the possible involvement of thiol groups and enzyme form in the inactivation of tyrosine aminotransferase and emphasize the need of elucidating the function of cysteine in the inactivation reaction.

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References