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Destabilization of tyrosine aminotransferase by amino acids

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Summary. Several L-amino acids (tyrosine, glutamate, methionine, tryptophan, and phenylalanine) and penicillamine destabilized purified tyrosine aminotransferase by removing enzyme-bound pyridoxal 5′-phosphate. The destabilization was measured as a progressive loss of enzyme activity in samples taken at intervals from a primary mixture that was incubated at 37°C. Each destabilizing amino acid either served as a substrate for this enzyme or was a product of transamination. In contrast, L-cysteine destabilized the enzyme only if liver homogenate was added, which generated polysulfide by desulfuration. Cysteine complexed free pyridoxal-5′-phosphate but did not remove it from the enzyme. Other amino acids did not destabilize tyrosine aminotransferase at the concentrations tested.

Keywords: Amino acids – Glutamate – Cysteine – Apoenzyme – Tyrosine aminotransferase

Abbreviations: TyrAT, tyrosine aminotransferase (E.C. 2.6.1.5); PLP, pyridoxal-5′-phosphate

Introduction

Tyrosine aminotransferase (TyrAT, E.C. 2.6.1.5) is unstable when incubated with homogenates of liver or kidney in the presence of a mixture of amino acids (Holten et al., 1967; Reynolds and Thompson, 1974; Reynolds, 1978). L-Cysteine accounts for most of the destabilization, which has been attributed to its ability to form a complex with pyridoxal-5′-phosphate (PLP), the cofactor for transamination (Holten et al., 1967; Pestana et al., 1971). However, TyrAT is inactivated by a product of desulfuration generated from cystine or cysteine by γ-cystathionase, which is abundant in the soluble fraction of liver (Hargrove and Wichman, 1987; Hargrove, 1988). While studying effects of sulfur amino acids on TyrAT, we observed that purified TyrAT was stable in the presence of L-cysteine but was unstable in the presence of L-methionine (unpublished data).

This report shows that Met, Tyr, Glu, Phe and Trp destabilized purified TyrAT by generating apoenzyme in vitro; subcellular fractions from liver were not required. In contrast, cysteine did not remove PLP from TyrAT, and the enzyme was stable in the presence of cysteine unless fractions from liver were added that generated polysulfide from the amino acid.

Materials and methods

Preparation of tissue samples and TyrAT

Tissue samples were homogenized in 0.25 M sucrose that contained 10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, and 0.1 mM PLP; the 100,000xg supernatant was prepared as described (Hargrove, 1988). For routine use, TyrAT was purified 100-fold (specific activity, 30 units/mg protein) by heating rat liver cytosol to 65°C, followed by chromatography on DEAE-cellulose. To test for substrate specificity and resolution of cofactor, it was purified to a specific activity of 510 units/mg as described (Hargrove, 1990). Inactivation was measured by incubating *ca.* 100 milliunits of enzyme at 37°C in 0.25 ml of 0.1 M Tris HCl, pH 7.5, that contained 1 mM final concentration of the specified amino acids. Samples were removed at intervals for the assay of TyrAT activity (Granner and Tomkins, 1970).

Tests for apoenzyme and substrate specificity

Formation of apo-TyrAT in the presence of Glu, Asp, and Tyr was monitored both as the decrease in absorbance at 415 nm, which indicates loss of the Schiff base between PLP and TyrAT (Hayashi et al., 1967), and by assaying the enzyme with and without added PLP (Reynolds, 1982). The substrate specificity of TyrAT was tested using a coupled assay based on glutamate dehydrogenase (Rej, 1980). The reaction was performed in a total volume of 1 ml, with the following composition: 50 mM potassium phosphate, pH 7.6, 1.6 mM α-ketoglutaric acid, 0.1 mM pyridoxal 5'-phosphate, 5 mM β-NAD, 4 mM ADP, 0.65 mM iodonitrotetrazolium, 35 units of glutamate dehydrogenase (Boehringer Mannheim), 5 units of diaphorase (Sigma Chemical Co.), and either 2 mM Tyr or 20 mM of all other amino acids. The reaction was initiated by addition of purified TyrAT (1.8 units of enzyme with specific activity of 510 units/mg). The protein concentration of the samples was determined by the method of Lowry et al. (1951) with fraction V bovine serum albumin as a standard.

The production of metabolites of cysteine and cystine in liver cytosols was correlated with inactivation of TyrAT as follows. The enzyme was incubated at 37°C in duplicate, 1 ml mixtures that contained 2 mg of cytosolic protein from liver, 2 mM L-cysteine, 0.4 units of TyrAT, 0.1 M Tris HCl, pH 7.5, 1 mM EDTA, and 0.1 mM PLP. Depletion of cysteine sulfhydryl was measured with 5,5'-dithio-*bis*-(2-nitro)benzoic acid (DTNB), and accumulation of sulfide and formation of sulfane sulfur were measured as described previously (Hargrove, 1988). In a second experiment, 1 mM L-cystine was substituted for the cysteine.

Results

Destabilization of tyrosine aminotransferase by amino acids

When partially purified TyrAT was incubated at 37°C in 0.25 ml of 0.1 M Tris HCl, pH 7.5, it gradually lost activity if 1 mM Met, Tyr, Trp, Phe, or Glu was included (Table 1). Destabilizing amino acids were defined as those that caused a loss of 50% or more of the initial TyrAT activity within 4 h or less. In contrast, TyrAT was stable in the presence of other amino acids, including L-cysteine.

Table 1. Substrate specificity of tyrosine aminotransferase and ability of amino acids to remove cofactor *in vitro*

Amino acid	Activity ratio (Minus PLP/Plus PLP)	Glutamate formed (nmole/min)	Total enzyme activity at 2h with PLP [nmole/min (% of initial)]
Tyr	0.09	58	43 (52%)
Glu	0.07	–	26 (31%)
Phe	0.08	3.9	41 (49%)
Trp	0.14	2.6	37 (45%)
Met	0.35	3.8	55 (58%)
Ala	0.94	1.4	71 (86%)
Cysteine	0.95	1.2	79 (95%)
Cystine	0.92	–	34 (41%)
Gly	0.96	2.4	76 (91%)
Ser	0.98	1.6	74 (90%)
Thr	0.95	1.9	82 (99%)
Ile	0.94	1.2	83 (100%)
Leu	0.83	2.1	76 (91%)
Val	0.90	1.9	75 (90%)
Asn	0.91	2.6	74 (89%)
Asp	0.94	1.8	71 (85%)
Gln	0.90	1.8	69 (84%)
Arg	0.92	2.0	83 (100%)
His	0.93	2.0	80 (96%)
Lys	0.92	1.3	83 (100%)
Penicillamine	0.06	–	38 (49%)

Effects of amino acids on the stability of TyrAT and fraction of holoenzyme were tested at 1 mM concentrations as described in Materials and methods. For tests of substrate specificity, concentrations used were 2 mM for L-Tyr and 20 mM for other amino acids. The coupled assay measures the amount of glutamate formed and could not be applied to Glu.

Inactivation due to L-cysteine required co-incubation with factors from liver, and was reversed by addition of dithiothreitol (Fig. 1A). In contrast, inactivation during incubation of TyrAT with Glu, Met, or other amino acids did not require fractions from liver, and occurred at a slower rate if cytosolic protein was included along with the partially purified enzyme (Fig. 1B). Dithiothreitol (2 mM) reversed the inactivation caused by all of the destabilizing amino acids, which suggested that the loss of activity was not attibutable to proteolysis.

To determine whether destabilization was due to loss of PLP, TyrAT was incubated with the amino acids and samples were removed for assay of residual activity in the presence and absence of 0.1 mM PLP (Reynolds, 1982). This procedure gives an estimate of the fraction of apo- and holo-enzyme. Penicillamine was also tested in this experiment because of its established ability to react with PLP (Schonbeck et al., 1975). The amino acids such as cysteine and serine that did not generate apoenzyme also did not destabilize TyrAT (Fig. 2A). However, penicillamine and all destabilizing amino acids except cystine promoted a rapid decline in activity assayed in the absence of PLP, and produced a gradual decline in activity measured in the presence of 0.1 mM PLP (Fig. 2B).

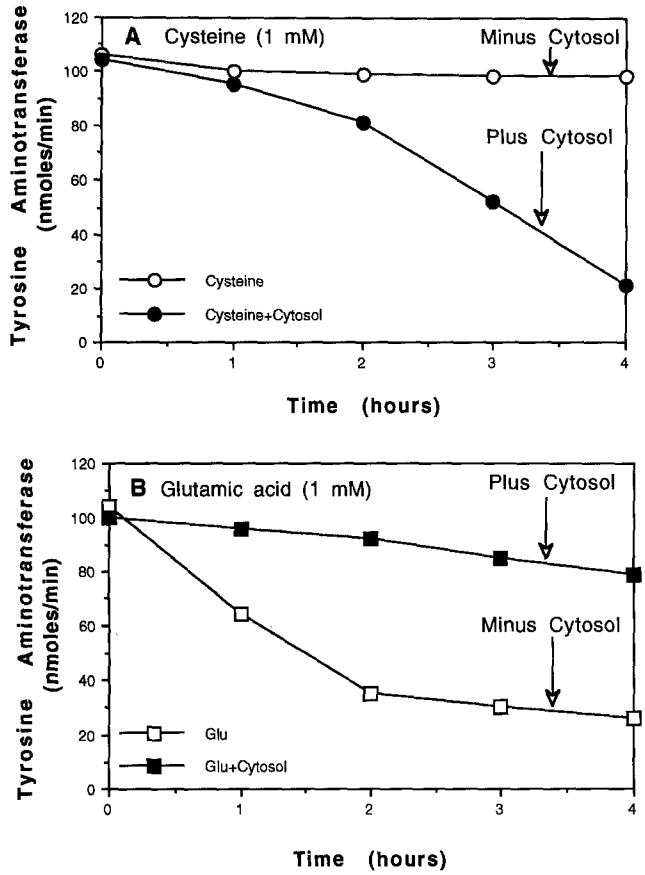

Fig. 1. Liver factors are required for the destabilization of tyrosine aminotransferase by cysteine but not glutamate. **A** Partially purified TyrAT was stable during incubation in 0.1 M Tris HCl, pH 7.5 in the presence of 1 mM L-cysteine alone (open circles), but was unstable when cysteine and a cytosolic fraction from liver were both added (closed circles). **B** In contrast, TyrAT was unstable during incubation with 1 mM L-glutamate (open squares) unless cytosol was added, in which case it was relatively stable (closed squares). Dithiothreitol (added at arrows) reactivated the enzyme. The assay for TyrAT used here measured total enzyme activity

The half-time for loss of activity when assayed in the absence of added cofactor was less than 5 min for Tyr, Glu, Phe, and penicillamine, but was 10 min for Trp and 30 min for Met. As the incubation continued, total TyrAT activity assayed in the presence of PLP also declined (last column in Table 1; numbers in parentheses give the percent of the initial activity at 2 h). According to this test, all of the destabilizing amino acids except cystine generated apoenzyme. Dithiothreitol did not affect the fraction of apoenzyme (not shown). Whereas penicillamine produced apoenzyme, L-cysteine did not as judged by assays performed in the absence of added PLP (Fig. 2A). L-Cysteine did form a complex with PLP, as confirmed by the shift in peak absorbance from 390 nm to 325 nm when the amino acid and coenzyme were incubated together in Tris HCl (not shown). When TyrAT was incubated in the presence of the other amino acids shown, its stability did not differ from the control with no added amino acids.

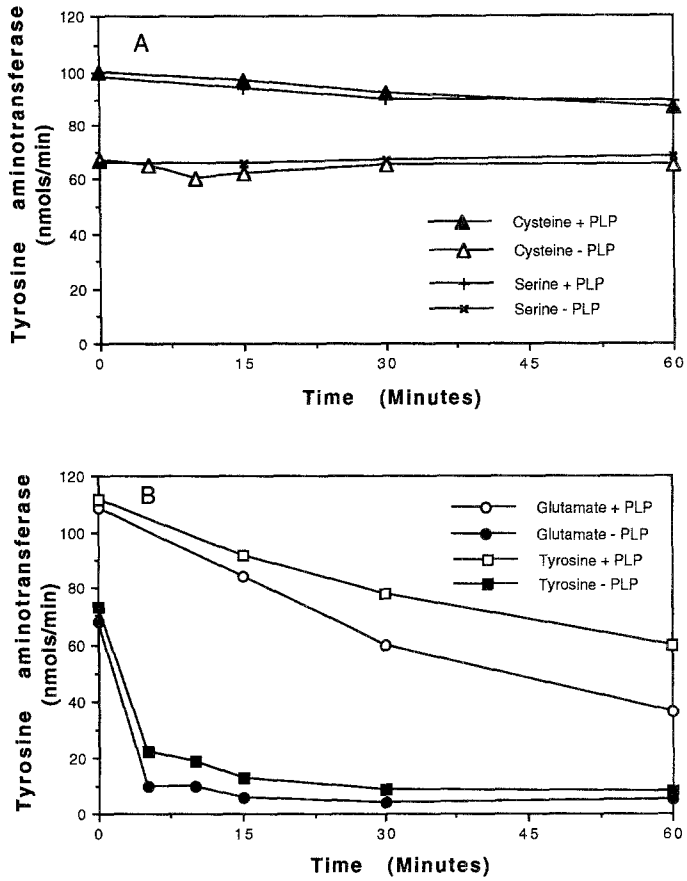

Fig. 2. Test for the ability of amino acids to generate apo-TyrAT. TyrAT was incubated with each amino acid as described in Materials and methods, and samples were taken for assay of residual enzymatic activity in the presence and absence of 0.1 mM PLP to estimate the ratio of apoenzyme to holoenzyme. **A** Results of the assay with and without added PLP are shown after a primary incubation with 1 mM L-cysteine (triangles) or L-Ser (crosses), which did not destabilize the enzyme. **B** In the presence of L-Tyr (squares) or L-Glu (circles), TyrAT was rapidly converted to apoenzyme (closed symbols), and total enzyme activity gradually declined (open symbols)

A general assay for transamination was used to test whether destabilizing amino acids also served as substrates for TyrAT (Rej, 1980). Phe, Trp, and Met each served as a substrate when employed at a concentration of 20 mM. Rates of transamination for these amino acids were 2.6–3.9 nmol/min, whereas values for other amino acids ranged from 1.2–2.6 nmol/min (Table 1). The possibility that higher concentrations of other amino acids might destabilize TyrAT was not tested.

Glutamate generates apro-tyrosine aminotransferase

The effect of Glu was of particular interest because this amino acid is a product of transamination, and is present in liver at a high concentration (Bloxam, 1972). To evaluate whether Glu generated apo-TyrAT, the enzyme was purified as

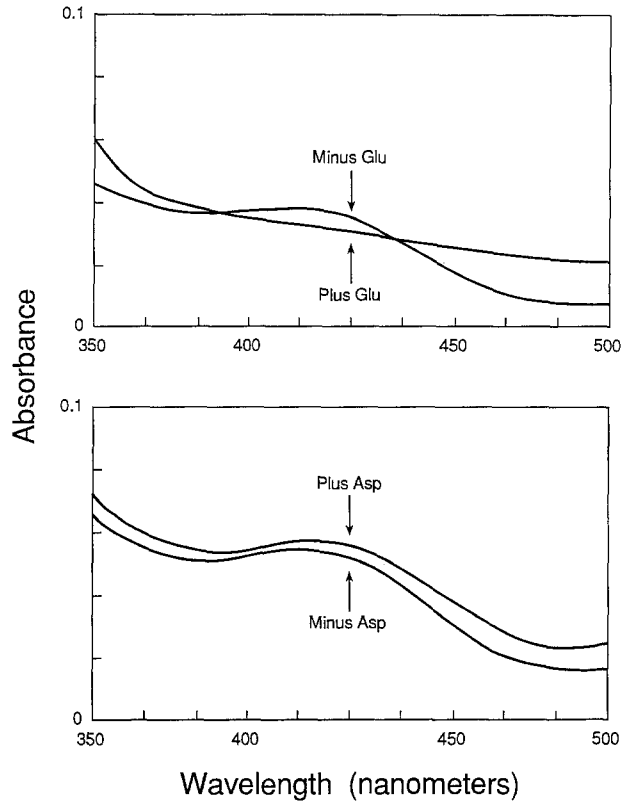

Fig. 3. L-Glutamate generates apo-TyrAT. The absorbance of purified holoenzyme (510 units/mg protein) was scanned between 350 and 500 nm prior to addition of L-Glu or L-Asp. Before the addition of Glu, the absorbance peak at 415 nm was prominent, but it disappeared after addition of 1 mM Glu (upper panel); no change was noted after addition of L-Asp (lower panel)

described in Materials and methods to a specific acitivity of 510 units per mg of protein, and the absorption spectrum was taken before and after treatment. The absorption peak at 415 nm decayed rapidly in the presence of Glu (Fig. 3) or Tyr (not shown), but remained during incubation with Asp. Asp did not destabilize TyrAT *in vitro*. The half-time for loss of the peak at 415 nm in the presence of Glu or Tyr was less than 5 min, in agreement with the ratio of activity measured in the presence and absence of PLP. These results indicate that Glu and Tyr both generated apoenzyme, whereas Asp did not. As another index that Glu generated apoenzyme, sensitivity of TyrAT to inactivation by iodoacetic acid was tested. Iodoacetate inactivated the enzyme much faster in the presence of Glu than minus Glu (data not shown). Agents that protected TyrAT against inactivation during incubation with Glu included dithiothreitol (2 mM), PLP (1.0 mM), α-ketoglutaric acid (10 mM), and potassium phosphate (20 mM). No inactivation occurred in mixtures containing combinations of these agents (not shown). Loss of enzyme activity was also pH dependent, and little inactivation occurred at pH 6 or 7, but TyrAT was unstable when incubated with destabilizing amino acids in Tris HCl at pH values of 7.5–8.5.

Destabilization due to cystine and cysteine

Unlike cysteine, cystine destabilized TyrAT in the absence of liver cytosol without generating apoenzyme. Incubation of TyrAT with 1 mM cystine at pH 8 in 25 mM Tris HCL caused loss of 60% of the enzyme's activity within 2 h, but the fraction of holoenzyme was unchanged (Table 1). Under these conditions, mixed disulfides form between cystine and TyrAT, and it is thought that the inactivation results from altered enzyme conformation due to formation of mixed disulfides (Federici et al., 1978). However, the inactivation that is observed when liver cytosol is included in the incubation mixtures results from a very different process. Under these conditions, there is a characteristic delay that occurs before inactivation begins. The sequence that is thought to produce inactivation with cysteine and cystine in the presence of liver cytosol is shown

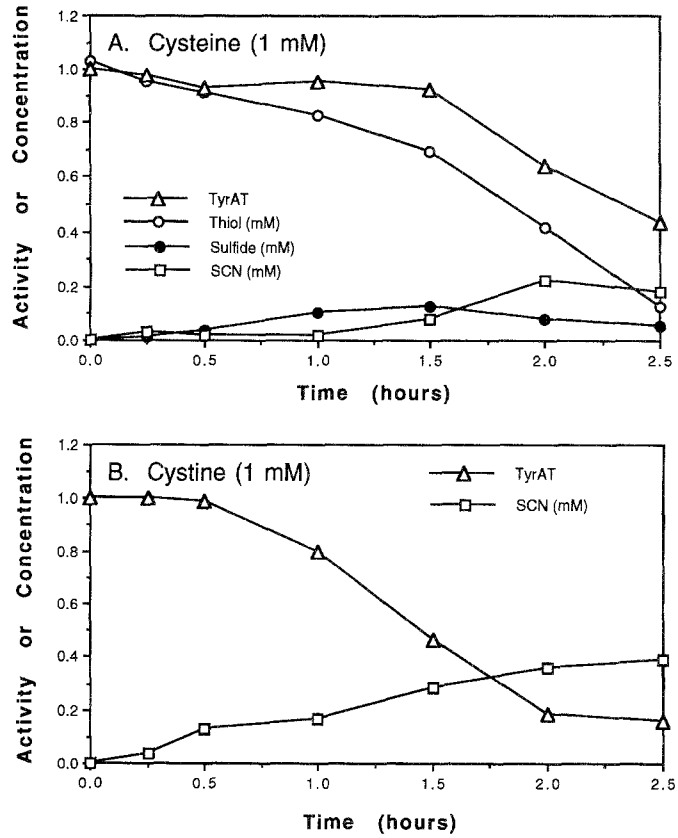

Fig. 4. Cysteine and cystine stimulate production of polysulfide in liver cytosols. **A** Inactivation of TyrAT was monitored during incubation at 37°C in 1 ml mixtures that contained 2 mg of cytosolic protein from liver and 2 mM L-cysteine. Depletion of cysteine thiol groups (open circles) was measured with DTNB, and accumulation of sulfide (closed circles) and formation of sulfane sulfur (open squares) were measured as described in Materials and methods. SCN indicates thiocyanate formed in the assay for sulfane sulfur, and the activity of TyrAT (open triangles) is expressed as a fraction of the initial value. **B** Accumulation of sulfane from 1 mM L-cystine with conditions as described in A except that L-cystine was substituted for L-cysteine. Note that in both cases, inactivation of TyrAT did not occur until much of the added amino acid had been converted to sulfane-containing products

in Fig. 4A and B, respectively. When cysteine was added to liver cytosol, its free sulfhydryl groups were oxidized during the course of the incubation as determined by assay with DTNB (Fig. 4A), but were stable during this period in a mixture lacking cytosol (not shown). During the first hour of incubation, the tubes smelled of sulfide, which accumulated to levels as high as 0.1 mM. Because hydrogen sulfide is volatile, the levels declined by 2 h of incubation (Fig. 4A). As the sulfide began to decline, polysulfide accumulated that could be detected as thiocyanate after treatment with sodium cyanide, and a precipitate formed that is thought to include inorganic sulfur and precipitated protein. Loss of TyrAT activity correlated with the loss of free thiol groups and the accumulation of polysulfide (which is indicated as SCN in Fig. 4A). Inactivation of TyrAT only occurred after the reducing equivalents had been exhausted, as indicated by the decline in sulfide and accumulation of polysulfide.

When cystine was included instead of cysteine, sulfide could not be detected (data not shown), presumably because there was very little free thiol. In this case, polysulfide could be detected within 15 min of incubation, and TyrAT activity began to decline within about 30 min as the sulfane sulfur accumulated. The delay in inactivation of TyrAT appeared to be related to the time needed for polysulfide to accumulate, which is longer with cysteine than with cystine (Fig. 4). This evidence suggests that inactivation of TyrAT in the presence of cysteine and cystine is unrelated to the production of apoenzyme, but occurs as a result of thiol oxidation.

Discussion

The present data suggest that amino acids can destabilize TyrAT in vitro by three mechanisms. Tyr, Glu, Met, Phe, Trp and penicillamine all generated apoenzyme according to the assay done in the absence of added PLP, and each of these compounds also destabilized TyrAT *in vitro* (Table 1). The apoenzyme was unstable, as demonstrated by a gradual loss of catalytic activity measured after addition of saturating levels of PLP to samples of the enzyme. The ability of DTT to protect against the loss of activity suggests that inactivation was due to oxidation of thiol groups. Other protective agents included PLP, α-ketoglutarate, and phosphate ion, which all bind in the catalytic center of transaminases (Arnone et al., 1985).

The destabilizing amino acids generated apoenzyme with the following order of effectiveness: Glu ≥ Tyr > Phe > Trp > Met. This order reflects the Michaelis constants for these substrates (Jacoby and LaDu, 1964; Hayashi et al., 1967) except for Met, for which no K_m has been published. The ability of Tyr to generate apo-TyrAT was originally reported by Hayashi et al. (1967), and Glu is known to remove coenzyme from several transaminases that utilize α-ketoglutaric acid as an acceptor for the amino group (Martinez-Carrion et al., 1985). The data shown in Figs. 2B and 3 demonstrate that Glu promotes release of the cofactor from TyrAT when incubated under conditions that favor the reverse half-reaction, and are consistent with earlier reports that the cofactor dissociates from TyrAT much more readily than from AspAT (Litwack and Cleland, 1968). By contrast, it is interesting that L-cysteine did not readily

generate apoenzyme whereas penicillamine did. Both compounds remove cofactor from some PLP-dependent enzymes (Pestana et al., 1971; Schonbeck et al., 1975), which may be partly explained by the ability of PLP to react with compounds that contain two nucleophilic groups to form stable, cyclic compounds called thiazolidines (Kallen et al., 1985). However, this feature alone does not facilitate dissociation of cofactor because PLP is covalently bound as a Schiff base to Lys 258 in AspAT and Lys 280 in TyrAT (Arnone et al., 1985; Hargrove et al., 1989). Compounds that promote dissociation must be able to orient in the catalytic pocket in a manner that permits the formation of free coenzyme as an external aldimine. Structurally, penicillamine differs from cysteine in that two methyl groups have replaced the hydrogen atoms on the β-carbon; one interpretation of the data is that these bulkier groups favor orientation of penicillamine's α-amino group towards the ε-amino group of Lys 280. The sulfhydryl group in penicillamine must facilitate this process, because the structurally similar branched-chain amino acids did not generate apo-TyrAT (Table 1), in contrast to the other large, neutral amino acids. Dissociation of cofactor is also favored by hydrolysis of the Schiff base in the external aldimine. This generates phosphopyridoxamine (PMP), which has a hundred-fold greater dissociation constant than PLP (Borri Voltattorni et al., 1975).

In contrast to the amino acids that produce apoTyrAT, cystine inactivates purified TyrAT by reacting with its free sulfhydryls to generate mixed disulfides (Federici et al., 1978, and personal observation). However, cystine is also a substrate for cystathionase, which is abundant in liver and possesses cystine desulfurase activity. The thiocysteine generated in the desulfurase reaction is very unstable, and promotes the inactivation of TyrAT and many other enzymes (Hargrove, 1988). Although cysteine is not a substrate for cystathionase, some cysteine is oxidized under the aerobic conditions used here, and the resulting cystine is converted to polysulfide. In the presence of cysteine, some polysulfide is reduced and hydrogen sulfide is generated (Fig. 4A), so that the addition of cysteine or cystine to liver cytosols results in enzyme inactivation. Proteolysis is not involved in this mechanism, because cystine-dependent inactivation of TyrAT does not alter the size of enzyme subunits as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (JLH, unpublished data). In addition to the cystathionase-dependent mechanism, there is evidence for an alternate form of cysteine-dependent inactivation in liver. Reynolds and Thompson (1974) reported that liver homogenates irreversibly inactivated TyrAT in the presence of cysteine but not cystine. Inactivation was stimulated by structural analogs of cysteine such as cysteamine and β-mercaptoethanol, as well as several catecholamines. In contrast to the present work, tyrosine and phenylalanine were not effective, and it is not clear what mechanism was responsible for the inactivation in that complex system.

Amino acids are normally present in the soluble fraction of liver; although the concentration of Tyr in liver is in the range of 50 nmoles per gram, the concentration of Glu may exceed 1 μmole per gram (Bloxam, 1962; Gross et al., 1991). A significant fraction of TyrAT is thought to be present as the apo-enzyme in vivo; estimates range from 20–60% (Reynolds, 1982). It will be interesting to learn whether Glu affects the amount of apoTyrAT in liver, and whether this

has any role in the rapid rate of degradation of TyrAT. Whether or not Glu and other amino acids promote dissociation of PLP from this enzyme in vivo, treatment of TyrAT with Glu is an effective means of generating apoenzyme for biochemical studies.

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