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Isolation and characterization of active N-terminal truncated apo- and holoenzyme of mammalian liver tyrosine aminotransferase

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Limited proteolysis was used to probe the structure of the apo- and holoenzyme of rat liver tyrosine aminotransferase Both were subjected to trypsinolysis and the major fragments were isolated and characterized Trypsin cleaves the apoenzyme after residues Arg^{57}, Lys^{64}, and Lys^{71} and the holoenzyme after Arg^{37} and Lys^{38} The difference in the accessibility of the enzyme deprived or associated with pyridoxal 5'-phosphate reflects two distinct conformations The activity, the affinity for the ligands and the thermostability of the purified truncated enzyme forms are similar to those of the native apo- and holoenzyme A model for the domain structure of mammalian tyrosine aminotransferase and a mechanism for its rapid turnover are proposed

Tyrosine aminotransferase, Rat liver, Structure, Proteolysis, Degradation, Structure/function relationship

1 INTRODUCTION

In mammalian liver, cytoplasmic tyrosine aminotransferase (TyrAT; EC 2 6 1 5) catalyzes the first step of the catabolism of tyrosine The transamination reaction involves the formation of a Schiff base between the carbonyl group of the coenzyme pyridoxal 5'-phosphate and alternately the ε-NH_2 group of a lysine in the catalytic site or the α-NH_2 group of the amino acid substrate [1] The rate of tyrosine degradation is regulated by the intracellular concentration of active TyrAT The biosynthesis of the enzyme can be increased by stimulation of gene transcription promoted by glucocorticoids (see [2] for a review on TyrAT) When induction is stopped, TyrAT concentration rapidly returns to the basal level because the protein has a short half-life ($t_{1/2}$= 2 h) when compared to non-inducible liver proteins (average $t_{1/2}$ = 2–3 days). Although several investigations were devoted to the mammalian liver enzyme, no information is available about its three-dimensional structure and the structural parameters determining its instability.

This paper describes a comparative study of the regions of the apo- and holoenzyme of TyrAT accessible to trypsin and the analysis of the structural and functional properties of the purified fragments. The results are interpreted in the frame of the structure/function relationship in this enzyme Their possible physiological significance in terms of a degradation mechanism is discussed on the basis of a comparison of the structure of TyrAT with that of other aminotransferases

Abbreviations TyrAT, tyorsine aminotransferase, PLP, pyridoxal 5'-phosphate

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2 MATERIALS AND METHODS

Native rat TyrAT was purified from an overproducing *E coli* strain [3] The dimeric holoenzyme (subunit M_r = 50 000, 454 amino acids [4]) has a specific activity of 420 ± 50 units/mg (one unit catalyzes the production of 1 μmol of *p*-hydroxyphenylpyruvate per min at 37°C under standard conditions [5]) SDS-PAGE was performed according to [6] The automated Edman-Chang method was used for analysis of N-terminal sequences in an Applied Biosystems sequencer C-terminal residues were identified using an Applied Biosystems Derivatizer/Analyzer, after cleavage of 1 nmol TyrAT (20 h at 37°C in 50 mM ammonium bicarbonate, pH 8 0, containing 0 1 mM diisopropyl fluorophosphate and 0 1% SDS) by carboxypeptidase B (dialyzed in 10% w/v lithium chloride solution) at a protease/TyrAT ratio of 1 4 w/w The apoenzyme was prepared by incubating 55 μM holoenzyme with 11 mM tyrosine in 100 mM potassium phosphate, pH 10 (final volume 360 μl), followed by size-exclusion chromatography on an analytical HPLC column (Protein Pak 300SW, i d 7 5 mm × 300 mm length, Waters) equilibrated with 200 mM sodium sulfate and 20 mM potassium phosphate, pH 6 5 Fractions containing protein were pooled and concentrated over Centricon-30 devices (Amicon) The labile apoenzyme was used extemporaneously and the holoenzyme was regenerated by adding a 10-fold excess of PLP Contamination of the apoenzyme by the holoenzyme and reciprocally did not exceed 10% as determined by absorbance at 330 nm Limited proteolysis with trypsin was performed in microcentrifuge tubes, each containing 1 5 mg TyrAT in 200 μl of 50 mM ammonium bicarbonate, pH 8 0 (protease/transaminase ratio = 1/500 w/w, 4 h at 37°C) High-M_r tryptic fragments were purified by size-exclusion chromatography as above Other biochemical methods were essentially those described previously [3] Trypsin (sequencing grade) and carboxypeptidase B were purchased from Boehringer The data bank 'Swissprot' and the software package from University of Wisconsin were used to search for homologies in primary structures, to predict secondary structures, and plot hydropathy profiles

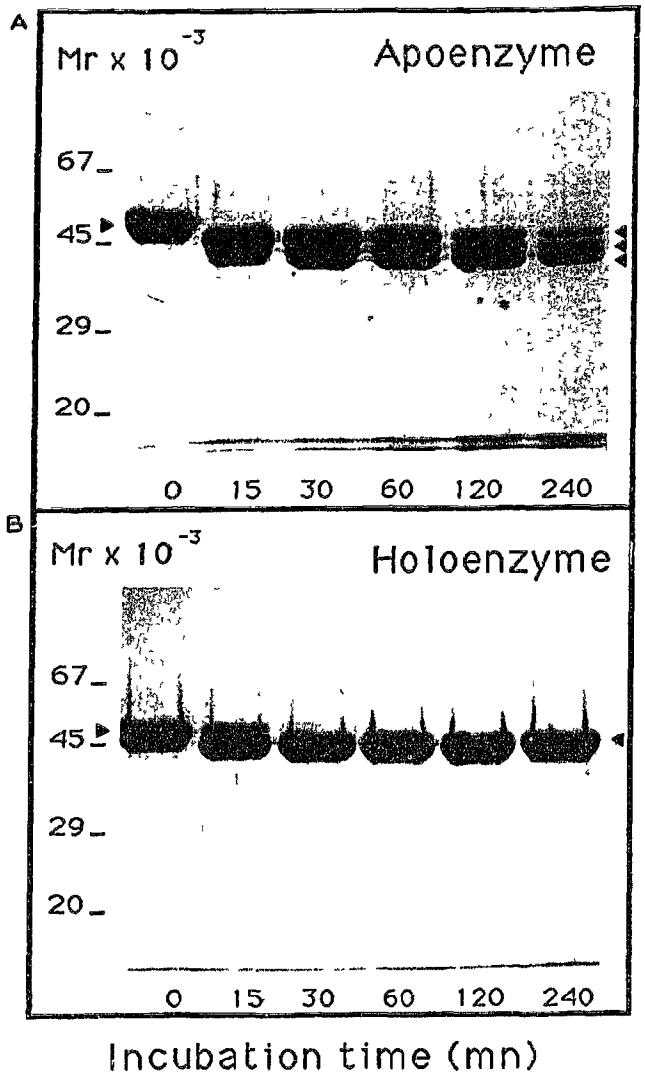

Fig 1 Kinetics of trypsinolysis of apo- and holoenzyme analyzed by SDS-PAGE Samples containing 5 μg protein were analyzed in 8 × 10 cm^2 gels

3. RESULTS

The presence of several basic residues in the N- and C-termini of TyrAT pointed out trypsin as a possible probe for its structure Six enzyme forms were characterized in this study because the native and the truncated TyrAT can exist as apo- and holoenzyme The trypsinolysis of the subunits of the apoenzyme generates polypeptide chains of variable length that are slowly hydrolyzed to peptides with M_r<18 000. Three major chains of M_r 42 000–45 000 are revealed by SDS-PAGE (Fig. 1A). The decrease of the amount of high-M_r polypeptides is accompanied by an increase of low-M_r peptides. Fragmentation of the holoenzyme rapidly leads to the accumulation of a stable polypeptide of M_r 46 000 (Fig. 1B) even when trypsin concentration is increased 10-fold (result not shown). During proteolysis, the activity of the holoenzyme is unaltered even after prolonged incubation whereas that of the apoenzyme decreases (Fig. 2). This inactivation superposes to the decrease of the amount of large fragments

The high-M_r peptides generated by the trypsinolysis of both enzyme forms were purified at the 10 mg scale by high performance size-exclusion chromatography and their structural and functional properties were analyzed Size-exclusion chromatography on a calibrated column indicates that the fragments are dimers of apparent M_r= 97 000 ±5000 (having an apparent hydrodynamic radius R_h = 40±2 Å and a frictional ratio f/f_0 = 1 5±0 1) but their shape is less elongated than that of native TyrAT (for which apparent M_r =160 000 ± 10 000, R_h = 47 ± 2 Å, and f/f_0 = 1 8 ± 0 1) The catalytic constants of the large tryptic fragments of both holo- and apoenzymes do not significantly differ from those of native TyrAT k_{cat} = 300 ± 50 s^{-1}, K_m for tyrosine 2 2 ± 0 5 mM, phenylalanine 480 ± 50 mM, tryptophan 50 ± 5 mM, α-ketoglutarate 0 8 ± 0.1 mM As for native TyrAT, no K_m for PLP could be obtained because kinetics are non-michaelian (V_{max} is obtained when 2 mol of PLP are present per dimer) [3]

The stability of the fragments of TyrAT was investigated by studying the resistance to heat denaturation. Indeed, PLP confers a high thermostability to native TyrAT ($t_{1/2}$>>12 000 min at 55°C) when compared to the apoenzyme ($t_{1/2}$ 1–3 min at 55°C) [3] Like the latter, all cleaved apoenzymes have very short half-lives in the presence or absence of tyrosine ($t_{1/2}$=1–2 min) and are only slightly protected by α-ketoglutarate ($t_{1/2}$=10 min). Addition of PLP renders these forms as stable as the native holoenzyme ($t_{1/2}$>>12 000 min at 55°C) and the trypsinolyzed holoenzyme has the same stability Microsequencing shows that about 9/10 of truncated apoenzyme molecules start with either Ala^{58}, Val^{65} or Thr^{72} (Table I) thus confirming the heterogeneity observed in electrophoresis (Fig 1A) The amount of individual polypeptides within the mixture varies with the extent of proteolysis For the holoenzyme only two major polypeptides starting at Lys^{38} or Ala^{39} are found (Table I)

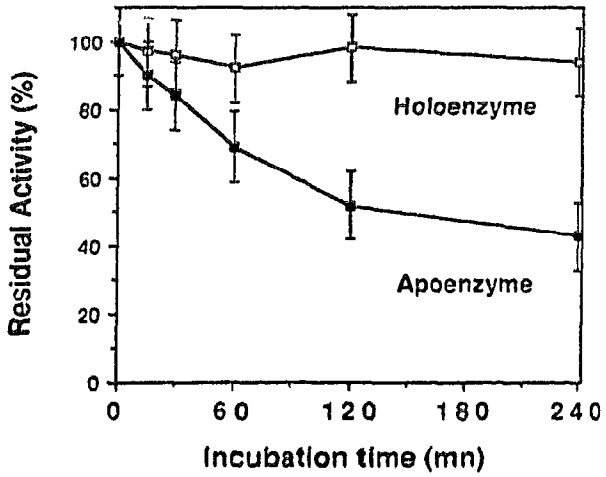

Fig 2 Variation of TyrAT activity during trypsinolysis of apo and holoenzyme Error bars correspond to an experimental error of 10%

Table I

N-Terminal sequences of the tryptic fragments of apo- and holoenzyme of TyrAT

Tryptic fragments[a] analyzed on blots (1) or in solution (2)		Amino acid sequences[b]	Approximate amount
Apoenzyme		*58*	
	(1)	A I V D N M	
		65	
	(1)	V Q P N P	9/10[c]
		72	
	(1)	T V I S	
		58 *65* *72*	
	(2)	A I V D N M K V Q P N P N K	
	(2)	V Q P N P N K T V I	9/10[c]
	(2)	T V I S L S I G D P	
		41	
	(2)	W D V R P S D M	1/10
	(1,2)	Possibly also blocked[d]	
Holoenzyme		*38*	
	(1)	K A R W D	9/10[c]
	(1)	A R W D V	
		38	
	(2)	K A R W D V R P S D M S N K T	9/10[c]
	(2)	A R W D V R P S D M S N K T F	
		58	
	(2)	A I V D	1/10
		65	
	(2)	V Q P N P N K	
	(1,2)	Possibly also blocked[d]	

[a]Analyses were performed on the major tryptic fragments purified either by electrophoresis or by size exclusion chromatography (see Methods)
[b]The number of Edman degradation cycles was 5–7 when the protein (200–500 pmol) was immobilized on a membrane after electrophoresis as in [3] or 5–27 when the protein (1 nmol) was in solution Contamination of apoenzyme by holoenzyme, and reciprocally, was ≤1/10
[c]The amount of individual polypeptide chains varied with the extent of proteolysis but the major fragments represented together about 9/10 of the protein analyzed
[d]The N-terminus of native TyrAT is fully blocked
[e]Both sequences were in about equimolar amounts

They are present in about equimolar amounts and represent together about 9/10 of the analyzed protein (Table I) Lysine is found as the C-terminus of all forms of TyrAT (yield >80%) but in all cases it becomes accessible to carboxypeptidase B only after denaturation of the protein

4. DISCUSSION

The present study was performed on native unproteolyzed rat TyrAT overproduced in and isolated from *Escherichia coli* cells [3] The difference in the kinetics and in the end products of the limited proteolysis of the apo- and holoenzyme by trypsin demonstrates the existence of two conformational states in TyrAT In the absence of PLP, its residues 39–71 are accessible to trypsin whereas association with the coenzyme masks this part of the polypeptide chain and only exposes the first 38 residues (particularly the hydrophilic region around Arg^{37}–Lys^{38}) (Fig. 3). The first 71 residues are not essential for ligand binding or catalysis The following four domains are postulated as elements of a working model of the structure of TyrAT (see Fig. 3). (i) An N-terminal domain limited to the extension 1–38 found so far only in cytoplasmic liver TyrAT [7] and alanine aminotransferase (there is 25% strict homology between them) [8] It contains two segments homologous in the rat [4] and human enzymes [9] (Fig 3) that might be involved in a specific function, e g the compartmentalization of TyrAT or its interaction with other metabolically linked enzymes [10] (ii) An intermediary segment (39–71) containing the highly conserved Pro^{55} and Lys^{71} (Fig 3) that seems not to be involved in subunit dimerization. Here TyrAT differs from mitochondrial and cytoplasmic pig aspartate aminotransferase holoen-

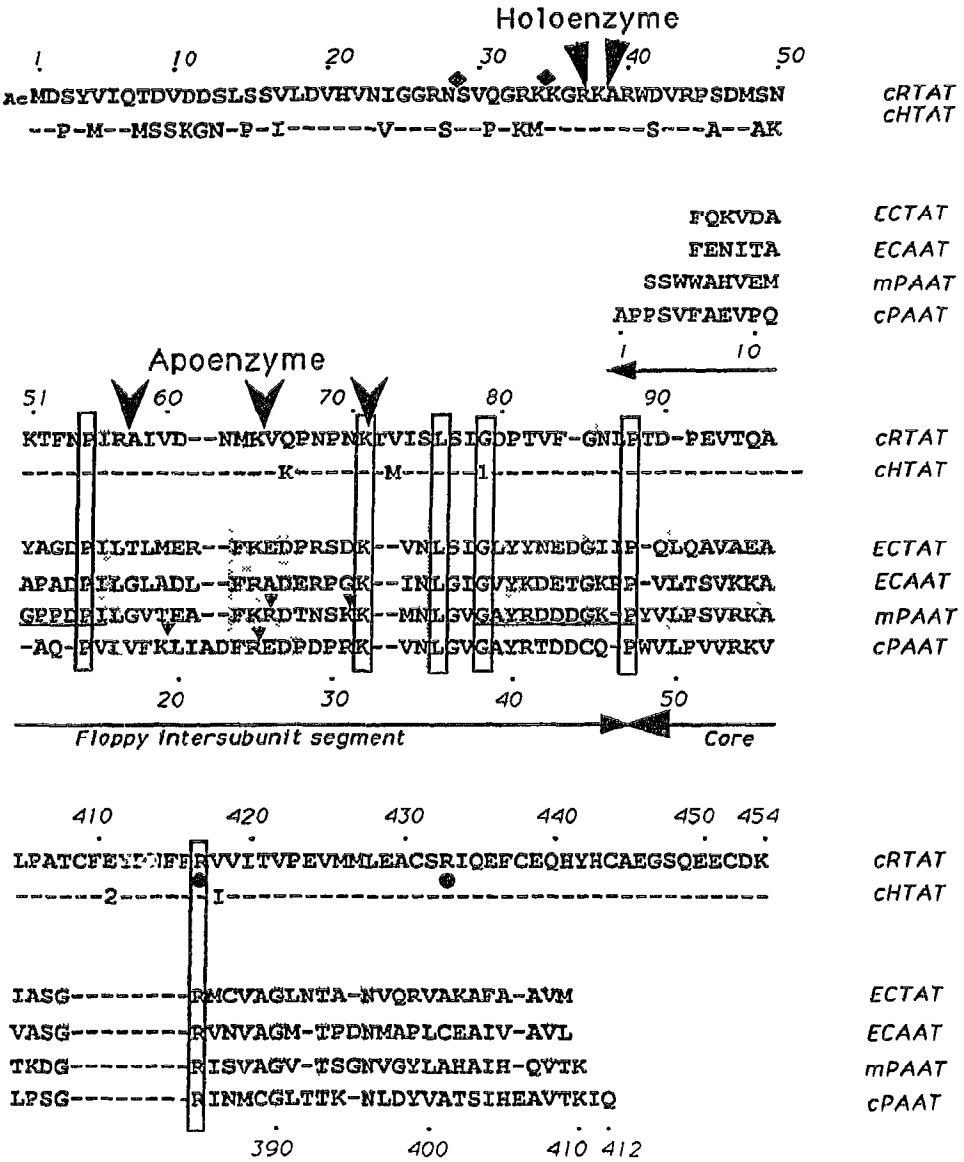

Fig 3. Tryptic cleavage sites in TyrAT and alignment of the N- and C-terminal regions of the primary structures of aminotransferases Large arrows indicate major cleavage sites in the apo- and holoenzyme Protein sequences are (cRTAT) cytoplasmic rat [4] and (cHTAT) human TyrAT [9], (mPAAT) mitochondrial and (cPAAT) cytoplasmic pig aspartate aminotransferase (taken from [7]), (ECTAT) aromatic amino acid aminotransferase from *E coli* [18] In human TyrAT, residues identical to those in rat TyrAT are replaced by hyphens and locations of introns 1 and 2 in the gene are indicated by numbers Upper numbering of residues corresponds to cRTAT sequence and lower numbering is that of cPAAT Highly conserved residues are boxed and residues aligned in more than four sequences are hatched Symbols are (▼) sites of tryptic cleavage in mPAAT and cPAAT, (♦) sites of cleavage by endogenous proteases at Asn^{28} and Lys^{14} in cRTAT [2], (●) two last arginines in the C-terminus that are not accessible to trypsin Hinge regions in chicken mitochondrial aspartate aminotransferase are underlined in the homologous sequence of cPAAT Limits of the floppy intersubunit domain and of the enzyme core in mPAAT [11] and cPAAT [12] are shown Alignments are as in [7] with minor adjustments

zymes that are inactivated by tryptic cleavage after Arg^{26}, Lys^{31}, and Lys^{19}, Arg^{75}, respectively (in the numbering system for aspartate aminotransferase) located in the floppy chain (19–47) extending between the subunits [11–14]. Deletion of residues 1–9 anchoring onto the neighboring subunit of the dimer decreases the affinity for the ligands (aspartate and 2-oxoglutarate), resistance to proteolysis and heat denaturation [15]. Since only TyrAT deprived of PLP can be cleaved in this region without leading to dissociation of subunits or inactivation, other structural elements stabilize its structure. (iii) A large core (72 to around 440) whose overall folding is most probably similar to that of aspartate aminotransferases according to primary structure alignments [7] and modeling studies [16]. In *E coli* aspartate aminotransferase this core (residues 47–329) folds autonomously as a monomer independently of the native N- and C-terminal extensions [17]. Chicken mitochondrial aspartate aminotransferase consists also of a large PLP-binding domain (Pro^{47}–Lys^{324}) and of a small domain

(encompassing Gly^{12}–Lys^{46} and Gly^{325} to the carboxyl end) [14] that rotates like a rigid body to close the active site upon binding of the substrates. This 'closed conformation' is opposed to the 'open conformation' of unliganded apo- or holoenzyme, and in this enzyme the presence of the coenzyme is not a prerequisite for the conformational change to occur. (iv) Finally, an 11 residue-long C-terminal extension found up to now only in cytoplasmic mammalian TyrAT (Fig. 3). The absence of homology in the end of known aminotransferases (Arg^{417} is the last conserved residue) suggests that this domain might be up to 36 residues long (Fig 3) Lys^{454} is not accessible to carboxypeptidase B in the active conformation of TyrAT and trypsin does not cleave after the two last basic residues Arg^{417} (which may be involved in substrate binding as deduced from sequence alignment with aspartate aminotransferases [7]) and Arg^{433} even when 71 residues have been removed at the N-terminus (Fig. 3).

Additional differences between TyrAT and aminotransferases of other specificity must be underlined TyrAT from rat and human and alanine aminotransferase from rat liver possess a high number of cysteines (16, 17 and 14, respectively) whereas this number is low in other aminotransferases [7]. For instance, bacterial aromatic amino acid aminotransferase [18] and aspartate aminotransferase have only 5 cysteines, and mitochondrial and cytoplasmic pig aspartate aminotransferases only 4 and 3, respectively In TyrAT all cysteines are clustered between positions 127 and 453 and consequently might be involved in the stabilization of the core and the C-terminal extension Finally, it may be questioned whether the difference in stability between the apo- and holoenzyme of TyrAT has any physiological significance and, in peculiar, if it could be involved in the rapid turnover of the enzyme Above results from in vitro experiments suggest that in vivo the removal of PLP and the cleavage in the region 40–70 might be the structural determinants that could switch TyrAT from a stable to an unstable conformation thus favouring its rapid degradation. In vitro both steps are required sequentially since PLP still protects the cleaved apoenzyme This possible multistep mechanism is in agreement with previous reports indicating that (i) the activity of TyrAT in vivo can be stabilized by administration of pyridoxin [19], whereas (ii) dephosphorylation of PLP by phosphatases [20] alters it, and (iii) the dissociation of the coenzyme could be the rate-limiting step conditioning the apoenzyme for proteolytic degradation [21]. Also, when group-specific proteinases participate in the degradation of some liver pyridoxal enzymes, the rate of inactivation depends on the rate of this conversion [22] It is postulated that in TyrAT cleavage in the region 41–71, whose accessibility is modulable by PLP, might unlock or sterically unblock the access to the PEST sequence [23]. According to sequence alignments, this stretch located in the C-terminal part of the polypeptide chain is most probably included in the small domain defined above for aspartate aminotransferase [14] Other structural features like e.g. the nature and acetylation of the N-terminal residue [24,25] or the phosphorylation of serine residues [2] might be involved in this mechanism

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