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PURIFICATION AND SOME PROPERTIES OF RAT LIVER TYROSYL-tRNA SYNTHETASE

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Summary

Rat liver cytoplasmic tyrosine:tRNA ligase (tyrosine:tRNA ligase, EC 6.1.1.1) was purified by ultracentrifugation, DEAE-cellulose chromatography and repeated phosphocellulose chromatography by more than 1500-fold. The molecular weight of the enzyme was approx. 150 000 as determined by Sephadex G-200 gel filtration. On the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the enzyme consisted of two subunits, each of 68 000 daltons.

We found the following K_m values for the enzyme: 13 μM for tyrosine and 1.7 mM for ATP in the ATP:PP_i exchange reaction and 13 μM for tyrosine, 210 μM for ATP and 0.14 μM for $tRNA^{Tyr}$ in the aminoacylation reaction. The rate of tyrosyl-tRNA synthesis was 50-fold lower than that of ATP:PP_i exchange. Addition of a saturating amount of tRNA did not affect the rate of ATP:PP_i exchange.

Introduction

Tyrosyl-tRNA synthetases (tyrosine:tRNA ligase, EC 6.1.1.1) have been isolated from bacteria [1—3], *Saccharomyces cerevisiae* [4,5] and hog pancreas [6]. Recently, Prasada Rao and Srinivasan [7] showed that purification of rat liver tyrosyl-tRNA synthetase yielded two protein fractions, with one fraction that catalysed tyrosyl-hydroxamate formation. Both fractions were required, however, for tyrosyl-tRNA synthesis. The authors concluded that this enzyme has an $\alpha\beta$ structure, which is of interest because no other amino acid activating enzyme has so far been found with this type of structure.

In this paper we describe a rapid method for the preparation of rat liver tyrosine activating enzyme without detectable decomposition into fractions. Some molecular and kinetic properties of the enzyme are also described.

Materials and Methods

Materials

Inbred PVG/c and WA rats (fAGUSf/Lac) purchased from MRC Laboratory Animals Centre were maintained in a specific pathogen-free (SPF) state. Male and female animals (100—300 days old) were used. DEAE-cellulose (DE 52) and phosphocellulose (P 11) were purchased from Whatman; Sephadex G-200 from Pharmacia Fine Chemicals; Fixion 2x8 anion exchanger chromatosheets were from Chinoin (Budapest); [U-^{14}C]tyrosine was from the UVVVR (Prague); [^{32}P]orthophosphate from the Isotope Institute (Budapest). [^{32}P]pyrophosphate was prepared as described by Loftfield and Eigner [8]. Synpor membrane filters (1.5 μm pore size) were from Chemapol (Prague); UM 10 Diaflo membranes from Amicon.

Tris, 2-mercaptoethanol, tyrosine, *p*-nitrophenyl-phosphate, Coomassie Brilliant Blue R 250, bovine serum albumin and catalase were purchased from Sigma; potassium acetate and *N,N'*-methylene bisacrylamide from British Drug Houses, sodium dodecyl sulphate (SDS) and ovalbumin from Serva. All other reagents were analytical grade commercial products.

Transfer ribonucleic acid (kindly provided by Dr. J. Nagy) was prepared from PVG/c or WA rat liver by the method of Rogg et al. [9]. One extinction unit (E_{260}) of tRNA mixture had 8—12 pmol tyrosine acceptor activity.

Escherichia coli RNA-polymerase (purified by the method of Burgess and Travers [10]) was the generous gift of Dr. A. Udvardy; rabbit muscle lactate dehydrogenase (kindly provided by Dr. E. Fejes) was purified according to Kornberg [11]; *E. coli* alkaline phosphatase was purified by the method of Torriani [12].

Purification of tyrosine:tRNA ligase

PVG/c rats were bled by intracardial punction during ether narcosis. Livers were homogenized according to the method of Ichimura and Tsukuda [13]. Cellular debris and nuclei were removed with centrifugation at 1000 × *g* for 10 min. After centrifugation at 96 000 × *g* for 90 min in a Beckman Model L-3 50 ultracentrifuge (rotor Type 42), the supernatant was thoroughly dialysed against 20 vols. buffer A. (Buffer A: 5 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol in 10 mM Tris/HCl (pH 7.2 at 25°C)).

Proteins were separated from nucleic acids using a DEAE-cellulose batchwise procedure. The dialysed supernatant was mixed with DEAE-cellulose equilibrated with buffer A (4 g wet weight DEAE-cellulose to 100 mg protein). After mixing for 30 min, the cellulose was filtered and washed with buffer A until the E_{280} value of the eluate decreased below 0.5. The cellulose was suspended in an equal amount of buffer A and poured into a column (3 cm diameter). Proteins were eluted with buffer A/0.1 M KCl. 10-ml fractions were collected; those containing the enzyme were diluted 4-fold and applied to a P 11 phosphocellulose column (1.5 × 4.5 cm, equilibrated with buffer A). The column was first washed with buffer A and then with buffer A/0.1 M KCl until the E_{280} value decreased to zero. Enzyme activity was eluted with buffer A/0.2 M KCl. 4-ml fractions were collected, concentrated by filtration through UM 10 diaflo membrane, diluted 4-fold with buffer A and applied to a column (1 ×

11 cm) of phosphocellulose. After washing with 25 ml buffer A, proteins were eluted with a linear gradient of 0—0.3 M KCl in buffer A. 2.5-ml fractions with the highest specific activities were pooled, concentrated as before and stored at −20° C after addition of an equal volume of glycerol.

Determination of the rate of pyrophosphate exchange

Tyrosine dependent ATP:PP_i exchange was determined in a reaction mixture (0.5 ml) containing 100 mM Tris/HCl (pH 7.65 at 37° C), 120 mM KCl, 1 mM 2-mercaptoethanol, 10 mM ATP neutralized with KOH, 15 mM $Mg(CH_3COO)_2$, 2.5 mM [^{32}P]$Na_4P_2O_7$ (4—7 cpm/pmol), 1 mM tyrosine and a limiting amount of enzyme. The reaction was started after 2 min preincubation at 37° C by the addition of enzyme. 50-μl aliquots were pipetted into 50 μl 0.1 M EDTA after different incubation times. Radioactive pyrophosphate and ATP were separated using Fixion 2x8 anion exchanger chromatosheets according to the method of Dusha and Dénes [14]. Radioactivity was determined by Cerenkov radiation in a Packard Tri-Carb liquid scintillation spectrometer.

Reaction mixtures without tyrosine were used as controls. One unit (U) of enzyme is defined as the amount required to incorporate 1 nmol pyrophosphate into ATP per min at 37° C under the standard conditions.

Determination of the rate of tyrosyl-tRNA synthesis

The reaction mixture (0.5 ml) contained 100 mM potassium cacodylate (pH 6.6 at 37° C), 15 mM $Mg(CH_3COO)_2$, 10 mM ATP neutalized with KOH, 1 mM 2-mercaptoethanol, 0.1 mM [^{14}C]tyrosine (10 Ci/mol), rat liver tRNA mixture with 0.8—1.5 μM tyrosine acceptor activity and a limiting amount of enzyme. After preincubation for 2 min at 37° C, the reaction was started by the addition of ATP. 0.1-ml aliquots were removed at different intervals and pipetted into 1 ml ice-cold 10% trichloroacetic acid. The precipitate was collected on a Synpor membrane filter and washed with 20 ml ice-cold 5% trichloroacetic acid solution. Filters were dried and radioactivity was measured in 10 ml scintillation cocktail (42 ml Liquid Scintillator NE 592 in 1000 ml toluene). The efficiency of counting ^{14}C on the filters in a Packard Tri-Carb liquid scintillation spectrometer was 80%.

Other methods

Gel filtration was carried out according to Andrews [15] using a column of Sephadex G-200 (1.5 × 92 cm) equilibrated in buffer A/0.1 M KCl. Elution was carried out with the same buffer. 2-ml fractions were collected. Molecular weight standards were bovine liver catalase (molecular weight 230 000—250 000), rabbit muscle lactate dehydrogenase (130 000—140 000), *E. coli* alkaline phosphatase (75 000—80 000) and horse heart muscle cytochrome *c* (12 400).

Purity and subunit molecular weight were examined by electrophoresis in 5% polyacrylamide gels containing 0.1% SDS according to the method of Shapiro et al. [16]. Cytochrome *c*, ovalbumin (44 000—46 000), bovine serum albumin (65 000—67 000), and *E. coli* RNA-polymerase (subunit molecular weights: 40 000, 95 000, 155 000 and 165 000) were used as standards. Purified tyrosyl-tRNA synthetase (2 μg) and standard proteins (1 μg per band)

were applied to a 17 × 16 × 0.25 cm slab gel. Proteins were separated at 25 V for 14 h using Shandon Southern DC electric power source. Gels were stained with 0.2% Coomassie Brilliant Blue R 250 in 50% CH_3OH/5% CH_3COOH for 2 h and destained with 7.5% CH_3COOH, 5% CH_3OH.

Protein concentration was determined by the method of Lowry et al. [17] using bovine serum albumin as standard.

Results

Tyrosyl-tRNA synthetase was purified approximately 1600-fold from the high-speed supernatant fraction of rat liver homogenates. A typical preparation is in Table I. During the homogenization procedure in 0.25 M saccharose, 3 mM $MgCl_2$ [13] neither nuclei nor mitochondria were disrupted; thus the isolated tyrosine activating enzyme is of cytoplasmic origin.

The enzyme required bivalent cations because it was inactivated by EDTA (unpublished data). 5 mM $MgCl_2$ in buffer A proved to be sufficient to preserve enzyme activity.

Phosphocellulose rechromatography is shown in Fig. 1. Although the combined fractions with highest specific activity were contaminated with other proteins, enzyme activity was approximately tripled. Purity after this step was tested in SDS polyacrylamide gel electrophoresis as shown in Fig. 2. 0.8 μg RNA polymerase α subunit was detected (bottom gel, Fig. 2). No contaminating protein band with similar intensity could be detected (top gel, Fig. 2).

The purified enzyme was tested for ribonuclease activity. ^{32}P-labelled RNA was incubated for 60 min with 20 times the amount of enzyme used in tyrosyl-tRNA synthesis. No detectable loss of trichloroacetic acid-precipitable radioactivity was found. We concluded that any ribonuclease contained in our tyrosine:tRNA ligase preparation did not interfere with the aminoacylation reaction.

The purified enzyme was stable for 6 months when stored at a final concentration of 0.05 mg/ml in a 1 : 1 mixture of buffer A and 87% glycerol at −20° C.

Molecular weight was estimated by Sephadex G-200 gel filtration. The molecular weight of the enzyme was calculated to be 150 000—160 000.

TABLE I

SUMMARY OF PURIFICATION OF TYROSINE-ACTIVATING ENZYME

The purification is described for 250 g rat liver. One unit (U) of tyrosyl-tRNA synthetase activity is the amount of enzyme that forms 1 nmol radioactive ATP per min according to standard conditions for tyrosine dependent ATP : PP_i exchange.

Fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Relative purification
Dialysed 96 000 × *g* supernatant	680	6400	73 000	11.4	100	1
DEAE-cellulose	40	428	30 650	71.3	42	6.3
Phosphocellulose I	4.0	1.3	11 360	8 740	15.5	767
Phosphocellulose II	2.0	0.25	4 550	18 350	6.23	1610

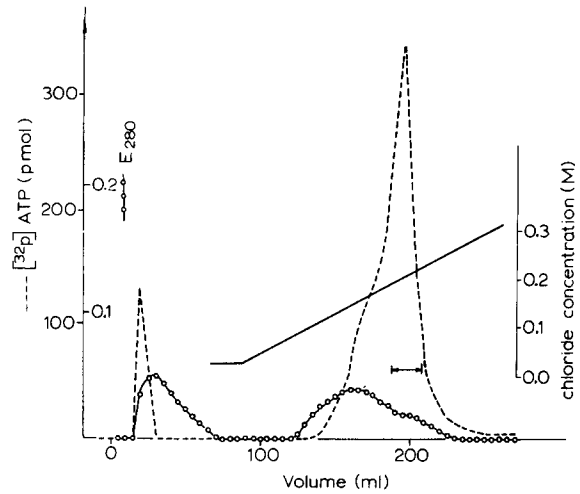

Fig. 1. Phosphocellulose rechromatography. 5120 units enzyme (0.8 mg protein) was applied to a phosphocellulose column of 1 X 11 cm, washed with 25 ml of buffer A (5 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol in 10 mM Tris/HCl (pH 7.2 at 25°C)) and eluted with 200 ml 0.0—0.3 M KCl in buffer A. 2.5-ml fractions were collected. Activity is expressed as the amount of radioactive ATP formed by 0.45 μl fraction in 6 min. 8 fractions from the second half of the activity peak (indicated by the arrows) were collected containing 2020 units enzyme and 0.11 mg protein.

SDS polyacrylamide slab gel electrophoresis revealed that tyrosine:tRNA ligase is slightly larger (68 000) than bovine serum albumin (65 000—67 000). These results indicate that rat liver tyrosyl-tRNA synthetase is probably a dimeric enzyme.

The pH dependence of ATP:PP_i exchange is shown in Fig. 3. Although the enzyme activity was slightly higher in potassium cacodylate buffer than in Tris/HCl, in our standard reaction mixture the latter was used because of its higher buffering capacity at the optimal pH.

Using saturating substrate concentrations (10 mM ATP, 2.5 mM PP_i, 15 mM Mg^{2+} and 1 mM tyrosine), the tyrosine dependent ATP:PP_i exchange was linear for 15 min or until 10% of radioactive PP_i was converted into ATP. Apparent Michaelis constants were determined as shown in Fig. 4 and Fig. 5 and were found 13 μM for tyrosine and 1.7 mM for ATP.

The pH dependence of tyrosyl-tRNA synthesis is shown in Fig. 6. Activity in potassium cacodylate buffer was much higher than in Tris/HCl buffer, probably because of the Tris-catalysed hydrolysis of tyrosyl-tRNA. In our

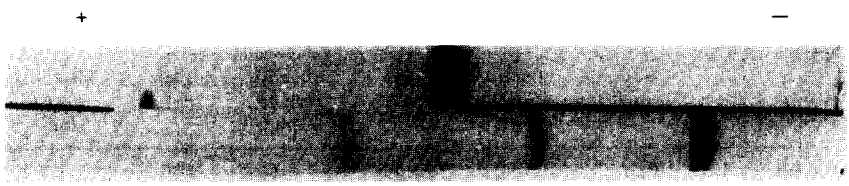

Fig. 2. Electrophoretic pattern of purified tyrosyl-tRNA synthetase from rat liver. Samples were run in 5% polyacylamide gels containing 0.1% SDS according to the method of Shapiro et al. [16]. Top gel: 10 μg protein after phosphocellulose rechromatography. Bottom gel: 5 μg E. coli RNA polymerase.

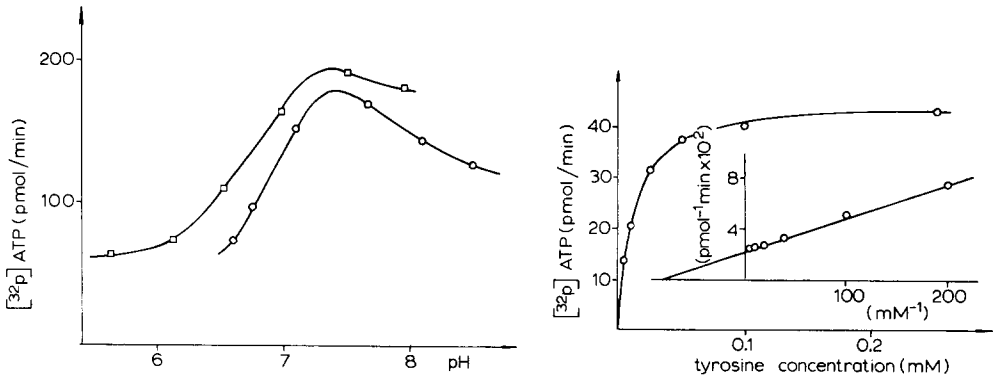

Fig. 3. pH-dependence of the ATP:PP_i exchange reaction. Initial rates were determined in standard reaction mixtures containing 16 U enzyme in either 0.1 M Tris/HCl buffer (o———o) or 0.1 M potassium cacodylate buffer (□———□).

Fig. 4. Effect of tyrosine concentration on the rate of ATP:PP_i exchange. Initial rates were determined in standard reaction mixtures containing 5 U enzyme and the indicated amounts of tyrosine.

standard aminoacylation mixture, 100 mM potassium cacodylate (pH 6.6) was used as buffer.

The tyrosyl-tRNA synthesis was linear up to 5 min in the presence of 10 mM ATP, 15 mM Mg^{2+}, 0.1 mM tyrosine and 1 μM $tRNA^{Tyr}$ as substrates. The rate of the aminoacylation reaction was only 2% of that of the ATP:PP_i exchange reaction. Apparent K_m values are 13 μM for tyrosine, 210 μM for ATP and 0.14 μM for $tRNA^{Tyr}$ as shown in Figs. 7, 8 and 9, respectively.

Addition of tRNA to the ATP:PP_i exchange mixture did not affect the reaction rate (Table II).

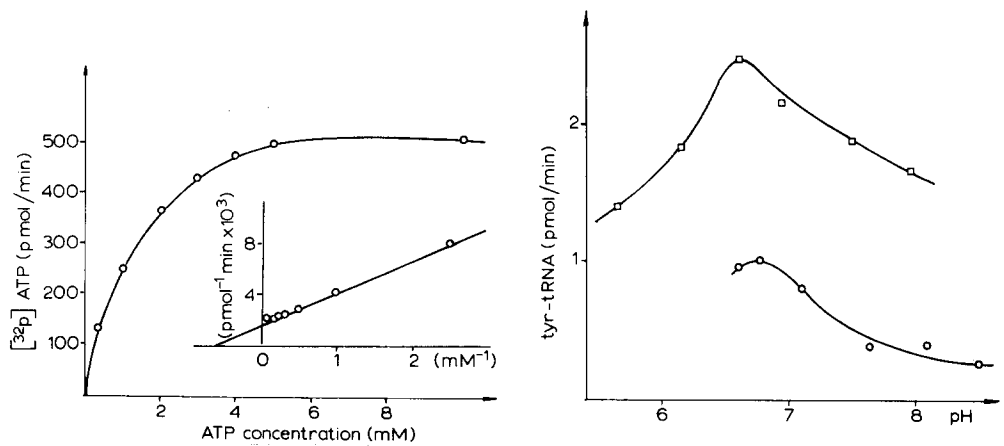

Fig. 5. Effect of ATP concentration on the rate of ATP:PP_i exchange. Initial rates were determined under standard conditions except for that increasing amounts of ATP:Mg (1 : 1) solution were added to reaction mixtures containing 5 mM PP_i, 5.5 mM Mg^{2+} and 50 U enzyme.

Fig. 6. pH-dependence of tyrosyl-tRNA synthesis. Initial rates were determined in standard aminoacylation reaction mixtures containing 0.7 U enzyme at the indicated pH values in either 0.1 M Tris/HCl buffer (o———o) or 0.1 m potassium cacodylate buffer (□———□).

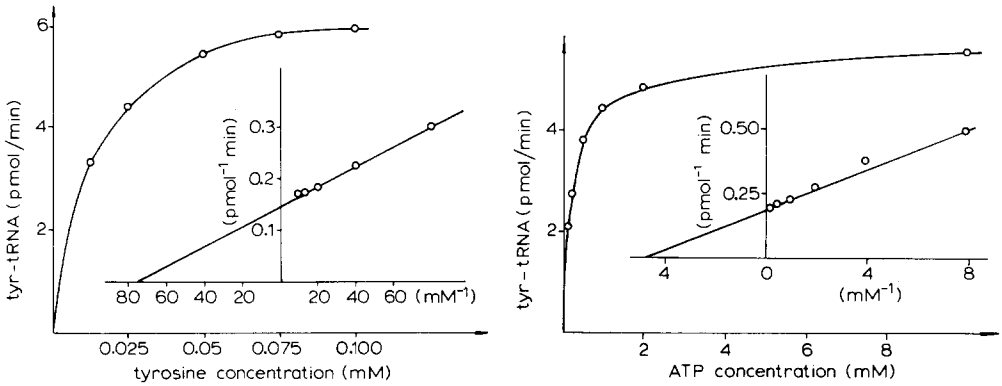

Fig. 7. Effect of tyrosine concentration on the rate of tyrosyl-tRNA synthesis. Initial rates were determined in standard aminoacylation reaction mixtures containing 1.5 U enzyme and the indicated amounts of tyrosine. Tyrosine acceptor activity of PVG/c rat liver tRNA was 1.4 μM.

Fig. 8. Effect of ATP concentration on the rate of tyrosyl-tRNA synthesis. Initial rates were determined in standard aminoacylation reaction mixtures containing 1.25 U enzyme and the indicated amounts of ATP. Tyrosine acceptor activity of WA rat liver tRNA was 0.9 μM.

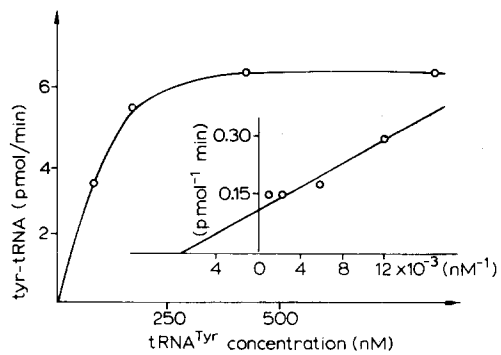

Fig. 9. Effect of tRNA concentration on the rate of tyrosyl-tRNA synthesis. Initial rates were determined in standard aminoacylation reaction mixtures containing 1.75 U enzyme and the indicated amounts of PVG/c rat liver tRNA. Tyrosine acceptor activity was 8.5 pmol per E_{260} unit of tRNA.

TABLE II

EFFECT OF TRANSFER RNA ON THE RATE OF ATP : PP_i EXCHANGE

Initial rates were determined according to standard conditions except for the addition of WA rat liver tRNA.

$tRNA^{Tyr}$ concentration (M)	ATP : PP_i exchange (pmol/min)	Rate (%)
0	42	100
$1 \cdot 10^{-8}$	42	100
$1 \cdot 10^{-7}$	42	100
$1 \cdot 10^{-6}$	40	95

Discussion

Rat liver cytoplasmic tyrosine:tRNA ligase was purified 1600-fold from postribosomal supernatant. Under these circumstances only one cytoplasmic enzyme specific for tyrosine was isolated. Although the enzyme was not entirely homogeneous, it was stable, free of detectable ribonuclease contamination and pure enough to allow determination of some of its characteristics.

Gel filtration of tyrosine:tRNA ligase after phosphocellulose chromatography gave a molecular weight of 150 000—160 000. Sumitting our purified enzyme to hydroxyapatite chromatography under similar conditions as described earlier [7], we did not achieve separation of factors A and B. The enzyme catalysing the complete ligase reaction, however, was eluted in two peaks corresponding to fractions 48—57 and 75—90. Each of the two peaks contained one half of the applied tRNA esterification activity and, after combining aliquots from the two peaks, the activity was additive. The enzyme obtained in both peaks gave similar SDS electrophoresis pattern and a 150 000 molecular weight. We conclude, therefore, that the tyrosine activating enzyme was not separated into inactive factors either during phosphocellulose or hydroxyapatite chromatography. The reason of separation of enzyme into active fractions in the latter step has not yet been elucidated.

Differences observed in the behaviour of the enzyme during hydroxyapatite chromatography could be ascribed to the different starting materials used in the two purification procedures (ref. 7 and this study). It is known that the postmitochondrial supernatant contains (among other proteins) all the ribosomal proteins, factors involved in the protein synthesis and the complex of amino acid activating enzymes [18] interacting with each other. The high ionic strength of $(NH_4)_2SO_4$ used for fractionation of this postmitochondrial supernatant could have resulted in the disruption of existing interactions between the proteins and also may have permitted the formation of new interactions. The observed weak interaction and peculiar stoichiometry between α and β subunits, purified by Prasada Rao and Srinivasan [7], permits such an explanation.

The fact that SDS polyacrylamide gel electrophoresis revealed one protein band of 68 000 molecular weight indicates that the enzyme consists of at least two, either similar or identical subunits. Tyrosine:tRNA ligases from *E. coli* [2] and *Bacillus stearothermophilus* [3] consist of two identical subunits. The monomeric enzyme from *S. cerevisiae* is active but has a tendency to form dimers [4]. A mutant of *S. cerevisiae* contains a tyrosine-activating enzyme with four identical subunits [5]. The rat liver enzyme was considered to have two non-identical subunits with the same molecular weight [7]. Our results permit this conclusion but further investigation is necessary to decide whether the subunits of the rat liver enzyme are different or not.

The specific activity of the maximally purified enzyme in ATP:PP_i exchange is 18 350 nmol/min/mg. Calculating with a mol. wt. of 150 000 and an estimated 80% purity, the turnover number in ATP:PP_i exchange is 3440 min^{-1}. This value is higher than that of the hog pancreas tyrosine-activating enzyme (1500 min^{-1}) [6], but lower than that of the bovine pancreas tryptophan activating enzyme (10 000 min^{-1}) [19]. The turnover number of rat liver tyrosine activating enzyme in the amino-acylation reaction is 69 min^{-1}. This

activity is higher than that of other rat liver amino acid activating enzymes isolated previously [20—23].

The rat liver enzyme (and the other known tyrosyl-tRNA synthetases) is able to catalyse ATP:PP_i exchange without tRNA. Using *E. coli* tyrosine-activating enzyme, Fersht and Jakes showed that the prior binding of tRNA greatly decreased the rate of tyrosyl adenylate formation [24]. In our experiments, however, addition of saturating amount of tRNA did not decrease the rate of rat liver enzyme catalysed ATP:PP_i exchange reaction to an extent greater than expected considering the maximal rate of aminoacylation reaction. We conclude that the formation of a complex catalysing ATP:PP_i exchange precedes the rate-limiting step of aminoacylation reaction.

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