# PURIFICATION AND SUBUNIT STRUCTURE OF TYROSINE AMINOTRANSFERASE FROM RAT LIVER CYTOSOL

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## 1. Introduction

The study of the molecular action of steroid hormones has been greatly facilitated by immunochemical techniques. In this respect, two glucocorticosteroid inducible liver enzymes, tyrosine aminotransferase (TAT) and tryptophane oxygenase (TO) have been studied extensively [1–4]. As most of the published methods of purification of TAT do not yield pure enzyme preparations we have attempted the purification of TAT from rat liver cytosol to homogeneity and the preparation of antibodies to the enzyme.

## 2. Materials and methods

### 2.1. *Buffers*

Buffer 1: 50 mM Potassium phosphate, 1 mM EDTA, 2 mM β-mercaptoaethanol, 0.2 mM pyridoxalphosphate, pH 6.5.

Buffer 2: 50 mM Potassium phosphate, pH 6.5, 1 mM EDTA, 2 mM β-mercaptoaethanol, 2.5 mM α-ketoglutarate.

### 2.2. *Immunoprecipitation of TAT in rat liver cytosol by antibodies to TAT*

The precipitation assay consists of 200 μl TAT-containing material, 1% Triton X-100, 1% sodium-deoxycholate, 0.9% NaCl, 50 mM EDTA and a sufficient amount of antibody to precipitate the enzyme. The amount of antibody has been determined before in separate experiments (10 μl = 0.2 $A_{280\ nm}$/approx. 1 enzyme unit precipitate).

### 2.3. *Enzyme assay*

TAT activity was determined by measuring the formation of *p*-hydroxyphenylpyruvic acid according to Diamondstone [5].

### 2.4. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was performed either in the absence of SDS according to Davis [6] or in the presence of SDS according to Weber and Osborne [7] or to Laemmli [8].

### 2.5. *Antibodies*

Antibodies to TAT I and TAT II were prepared by immunization of rabbits with the corresponding purified enzymes. The antisera were further processed for IgG by ammonium sulphate precipitation and by fractionation of the redissolved precipitate on Sephadex G-200 (see Pharmacia gel filtration manual).

### 2.6. *Countercurrent electrophoresis*

Countercurrent electrophoresis was performed as described in refs [9] and [10].

## 3. Results and discussion

### 3.1. *Purification of TAT*

As starting material we used rats pretreated with 20 mg cortisol/100 g body wt for 8 h. The initial purification steps, starting from the 100 000 g cytosol, involved ammonium sulphate precipitation, heat treatment, DEAE-cellulose chromatography and Sephadex G-200 gel filtration, performed as described by Valeriote et al. [11].

The specific activity of the enzyme preparation obtained up to this step was 300–500 units/mg protein, corresponding well to the results obtained by Valeriote et al. [11]. However, at this point of purification, the enzyme preparation was still contaminated by protein impurities, so that further purification was necessary (see fig.1). We therefore submitted the enzyme preparation to fractionation on DE-52 cellulose. The column was equilibrated with buffer 1 and eluted with a linear 50–30 mM KCl gradient. The active fractions eluting at 0.2 M KCl were further purified on a Sephadex G-200 superfine column (2 × 90 cm) equilibrated with buffer 1. Finally, the obtained enzyme preparation was submitted to chromatography on CM-50 Sephadex columns, as proposed by Johnson et al. [12]. As seen from fig.2, two enzyme activities were thus separated, enzyme I, with a specific activity of 1400 units/mg protein and enzyme II, having a specific activity of 750 units/mg protein.

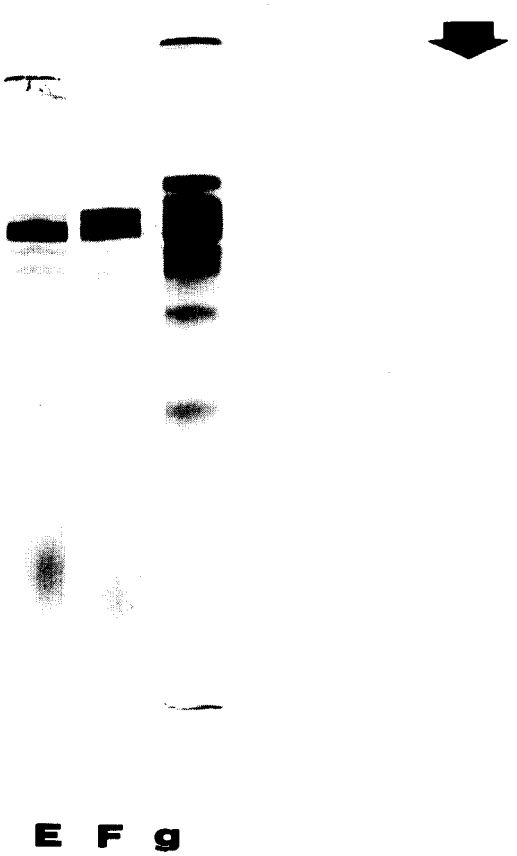


Fig.1. SDS–acrylamide electrophoresis according to Weber and Osborne [7] of different TAT preparations. E = TAT I, F = TAT II, g = TAT preparation purified up to the G-200 step of Valeriote et al. [11], omitting sucrose gradient centrifugation.

### 3.2. *Criteria of purity and subunit structure*

SDS–acrylamide electrophoresis of TAT I in the system of Weber and Osborne [7] reveals the presence of one main band corresponding to 95% of the total protein, having a molecular weight of 44 000, smaller than the heavy chain of IgG, which indicates the presence of two indentical subunits per enzyme molecule (fig.1). SDS–gel electrophoresis of TAT II reveals the presence of two main bands with molecular weights

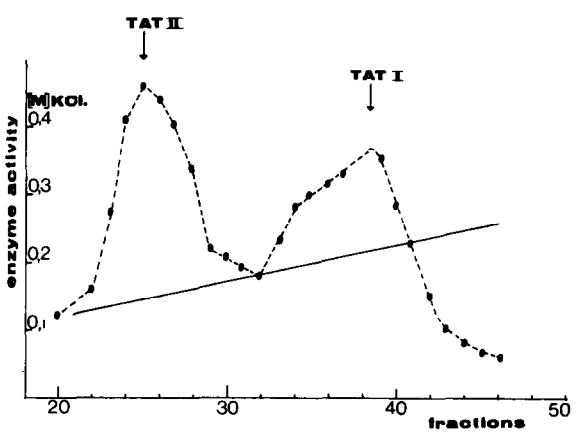


Fig.2. Separation of TAT I and TAT II by Sephadex CM-50 superfine chromatography. (●—●—●) Relative enzyme activity. (————) Linear KCl gradient. The active fractions obtained from the Sephadex G-200 superfine column have been pooled, supplemented with α-ketoglutarate (2.5 mM end-concentration) and applied to a 1 ml volume column. Gradient elution was performed with a linear KCl gradient (0–0. 35 M KCl in buffer 2) resulting in two active enzyme peaks [12]. Both peaks have been concentrated by Diaflo pressure-filtration to a concentration of more than 2 mg/ml and stored at -70°C.

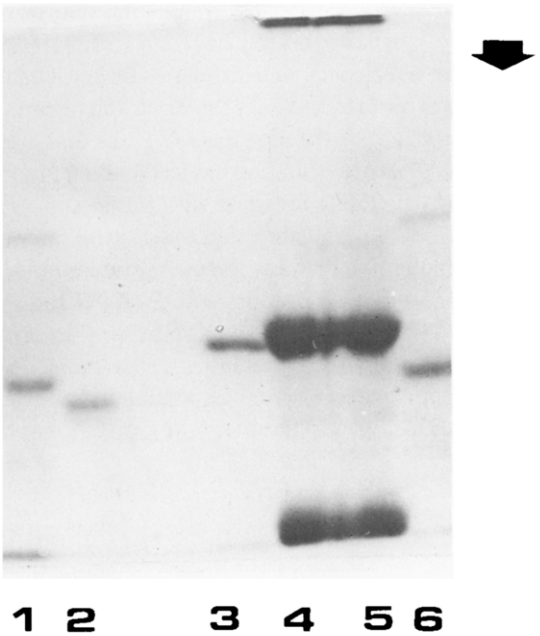


Fig.3. Acrylamide gel electrophoresis on gel-slabs according to Laemmli [8]. (1) = Ovalbumin. (2) = TO. (3) = TAT I. (4) = IgG. (5) = IgG. (6) = Ovalbumin.

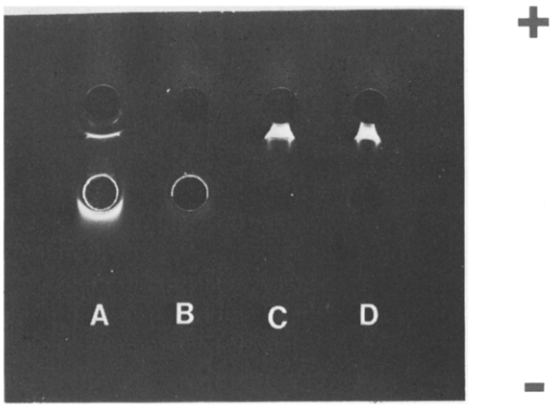


Fig.4. Countercurrent electrophoresis at pH 8.2. Antibody to TAT was applied to each well of the upper row. Wells of the lower row were filled with the following preparations: (A) Hepatic cytosol of cortisol treated rats. (B) Hepatic cytosol of untreated rats. (C) TAT I. (D) TAT II.

of 44 000 and 46 000 respectively, corresponding to about 95% of the protein. Acrylamide gel electrophoresis of TAT on Laemmli gel-slabs reveals that the enzyme comigrates with the heavy chain of IgG and has a slower mobility than marker ovalbumin (see fig.3).

Approximately 5% of both preparations consists of polypeptide contaminants with defined lower molecular weights. An increase on the amount of small polypeptides is observed if the enzyme preparations are kept at room temperature in the presence of the SDS-buffers.

### 3.3. *Specifity of the enzyme–antibody interaction*

The interaction of the antibodies with the enzymes result in complete enzyme inactivation.

Specifity of the antibody preparations is demonstrated by Ouchterlony double-diffusion tests and countercurrent electrophoresis (fig.4). It appears that both enzyme preparations (TAT I and TAT II) are immunologically identical as shown by the formation of identical precipitates in cross experiments.

As it is well known that the Ouchterlony test does not always discriminate antigens with similar molecular weights, we performed the following experiment in order to clarify whether the 46 000 band of TAT II is a modified TAT subunit or a contaminant.

[$^3$H] Leucine and cortisol have been injected into rats and the animals killed after 3 h and 12 h. Cytosol prepared from both animals were enriched in TAT by heat denaturation and batchwise DE-52 column chromatography and then immunoprecipitated with TAT I antibody. The immunoprecipitate was further submitted to polyacrylamide gel electrophoresis on Laemmli [8] slab-gels, which were then subjected to fluorography [13]. As seen from fig.5 only one labelled polypeptide band is evident which is comigrating with the 44 000 subunit. Thus both the enzyme induced after 3 h administration of cortisol as well as the 12 h induced enzyme have the subunit structure of TAT I. We can therefore draw the conclusions that the 46 000 component of TAT II has no common antigenic determinant with TAT I and represents contaminating material.

Our results show that the molecular weight of TAT I and TAT II is approximately 90 000 on the basis of Sephadex G-200 superfine gel filtration. TAT I and II show in SDS–gel electrophoresis one main subunit of 44 000, TAT II in addition a 46 000 band which, on the basis of the immunoprecipitation experiments described, is a contaminant. Both enzymes seem therefore to be composed of two subunits each, identical in respect

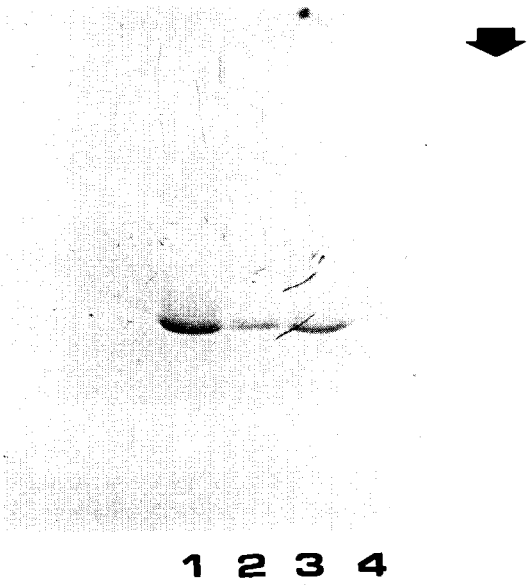


Fig.5. Fluorography of [$^3$H]leucine labelled immunoprecipitable TAT derived from cortisol induced animals. Two rats were injected with 20 mg/100 g cortisol. After 2 h rat A was injected with 500 $\mu$Ci, rat B with 2 mCi of [$^3$H]leucine (spec. act. 60 Ci/mmol). After 3 h and 12 h rats A and B respectively were killed. The cytosols was enriched for TAT activity and precipitated with antibodies against TAT I. The immunoprecipitates were washed, submitted to SDS–acrylamide gel electrophoresis and further to fluorography [13]. (1) = aliquot of immunoprecipitate of rat A cytosol. (2) = 5 × aliquot of immunoprecipitate of rat A cytosol. (3) = aliquot of immunoprecipitate of rat B cytosol. (4) = 5 × aliquot of immunoprecipitate of rat B cytosol.

to molecular weight. Iwasaki et al. [14] have determined the molecular weights of three forms of TAT by SDS–acrylamide gel electrophoresis. They found molecular weights 120 000 – 160 000 and conclude that, due to difficulties of protein dissociation under the conditions used, these values probably correspond to the undissociated enzyme molecules.

Using Sephadex G-200 gel filtration, they have estimated the molecular weight of the three forms to be approximately 107 000, a value closer to the one estimated by us.

Two other groups have determined the subunit structure of TAT. Bond [15] has found a subunit of 25 000 and a molecular weight of 100 000, whereas Auricchio et al. [16] a 31 000 subunit, the respective molecular weight of the enzyme being 115 000. Due to the presence of impurities in these preparations, these values should be regarded with caution.

Johnson et al. [12] suggested that, in the early stages of induction TAT I is the main protein induced, whereas later in the induction period TAT II and a further TAT form, TAT III, predominate. Our results exclude major modifications in respect to molecular weight as an explanation for the possible transformation of one enzyme form to another.

## References

[1] Litwack, G. (ed) (1972) Biochemical Actions of Hormones, Vol. II, Academic Press, New York and London.
[2] Ono, M., Inoue, H., Suzuki, F. and Takeda, Y. (1972) Biochim. Biophys. Acta 284, 285–297.
[3] Poillon, W. N., Maeno, H., Koike, K. and Feigelson, P (1969) J. Biol. Chem. 244, 3447–3456.
[4] Litwack, G. and Rosenfield, Sh. (1973) Biochem. Biophys. Res. Commun. 52, 181–188.
[5] Diamondstone, T. J. (1966) Anal. Biochem. 16, 395–399.
[6] Davis, B. J. (1964) Ann. NY Acad. Sci. 21, 404–409.
[7] Weber, K. and Osborne, M. (1969) J. Biol. Chem. 244, 4406–4412.
[8] Laemmli, V. K. (1970) Nature 277, 680–685.
[9] Gocke, D. J. and Howe, L. (1970) J. Immunol. 104, 1031–1032.
[10] Pesendorfer, F., Krassnitzky, O. and Wewalka, F. (1970) Klin. Wschr. 48, 58–59.
[11] Valeriote, F. A., Auricchio, F., Tomkins, G. M. and Riley, D. (1969) J. Biol. Chem. 244, 3618–3624.
[12] Johnson, R. W., Roberson, L. E. and Kenney, F. T. (1973) J. Biol. Chem. 218, 4521–4527.
[13] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
[14] Iwasaki, Y., Lamar, C., Danenberg, K. and Pitot, H. C. (1973) J. Biochem. 34, 347–357.
[15] Bond, J. S. (1971) Biochem. Biophys. Res. Commun. 43, 333–339.
[16] Auricchio, F., Valeriote, F., Tomkins, G. and Riley, W. D. (1970) Biochem. Biophys. Acta 221, 308–313.