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PURIFICATION AND SUBUNIT STRUCTURE OF MITOCHONDRIAL PHENYLALANYL-tRNA SYNTHETASE FROM YEAST

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SUMMARY : Yeast mitochondrial Phe-tRNA synthetase is purified about 380-fold to homogeneity by precipitation with ammonium sulfate and chromatography on DEAE-cellulose, on two hydroxylapatite and on two AH-Sepharose 4B columns. The yield is 17 %. The molecular weight is 266,000 as determined by Sephadex G-200 chromatography. The enzyme presents two types of subunits with molecular weights of 57000 and 72000, respectively, determined by urea-sodium dodecyl-sulfate gel electrophoresis.

INTRODUCTION : Mitochondria of Saccharomyces cerevisiae contain specific aminoacyl-tRNA synthetases for which the nuclear origin in several cases has already been reported (1).

Chromatographic and immunological differences between mitochondrial and cytoplasmic Phe-tRNA synthetases (2) indicate the existence of two different molecular species. In an attempt to shed further light on the question as to whether or not mitochondrial and cytoplasmic enzymes are physically different, we report in this paper an isolation and purification procedure of mitochondrial Phe-tRNA synthetase and evidence for its subunit structure. To our knowledge this is the first molecular report on mitochondrial Phe-tRNA synthetase.

MATERIALS AND METHODS

Saccharomyces cerevisiae strain ρ^+ IL8-8C was grown as described in (1). Preparation of mitochondria was performed according to (3). A sucrose gradient was used to purify further the mitochondria (4).

Phe-tRNA synthetase assays : Phe-tRNA synthetase activity was measured by the acylation reaction : the 0.1 ml reaction mixture contained 1 µmole ATP (Na salt), 1.5 µmole $MgCl_2$, 2.5 µmole Tris-HCl (pH 8), 0.5 µmole 2-mercapto-ethanol, $4A_{260}$ units of yeast tRNA (Boehringer, Mannheim, GFR), 5 nmole (12 µCi) of L - [^{3}H] Phenylalanine (CEA, Saclay, France). Incubation time was 15 min when testing column fractions or 1-5 min when measuring enzyme specific activity at 37°C. 80 µl reaction mixture were absorbed on a Whatman 3 MM paper disc (Ø 25 mm) which was then immerged in 5 % trichloracetic acid for 10 min at 4°C. Two washings in 5 % trichloracetic acid and two in pure ethanol at 4°C were then performed. Paper discs were counted in 5 ml of a liquid scintillation medium containing 4 g/l omnifluor (NEN, Frankfurt, GFR) in toluene.

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Protein determination : The protein concentration was determined by the method of Lowry (5) with bovine serum albumin as standard.

Gel electrophoresis : Analytical gel electrophoresis in the absence of sodium dodecylsulfate and urea was performed as described in (6) with 5 % acrylamide, in the Tris buffer system.

Gel electrophoresis with 0.1 % SDS and 4 mol/l urea, for molecular weight determination of subunits, was carried out according to (7) with 7 % acrylamide. Bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogene (25,700) and myoglobin (17,000) were used as markers.

Analytical gel filtration : Molecular weight determination of native enzyme was performed on a Sephadex-G 200 (Pharmacia, Uppsala) column (1.6 x 42 cm) previously calibrated with cytoplasmic Phe-tRNA synthetase (276,000) (8) purified and measured for activity as described in (9), E. coli β-galactosidase (130,000) and bovine serum albumin.

Purification procedure : All steps were carried out at 4°C. All buffers used contained 5 mmol/l 2-mercaptoethanol and 10^{-4} mmol/l phenylmethylsulfonyl-fluoride.

Crude extract : The pocedure of Accocebevry et al. (10) was used. Purified mitochondria were homogenized for 45 min in 10 mmol/l Tris-HCl (pH 8.5), 60 mmol/l KCl, 20 mmol/l $MgCl_2$, 1 % (v/v) glycerol. ATP was then added at a final concentration of 1 mmol/l. The suspension was stirred for 15 min and then 10 ml samples were disrupted using an Ultra Turrax, (2 min at position 120). 2-mercaptoethanol was added to the suspension at a final concentration of 10 mmol/l. The crude extract was centrifuged at 105,000 x g for 2h30.

Ammonium sulfate fractionation : The supernatant (crude extract) was brought to 70 per cent ammonium sulfate saturation at 4°C and left overnight at 4°C. The precipitate was collected by centrifugation at 58,000 x g for 45 min, dissolved in and dialyzed against 10 mmol/l Tris-HCl (pH 7.4), 15 mmol/l $MgCl_2$, 10 % (v/v) glycerol.

DEAE-cellulose chromatography : The dialyzed ammonium sulfate fraction was applied to a column (2.4 x 42 cm) of DEAE-cellulose (DE-11, Whatman, Balston), equilibrated with 10 mmol/l Tris-HCl (pH 7.4), 15 mmol/l $MgCl_2$, 10 % glycerol. The elution was performed with the same buffer at a flow rate of 42 ml/h. Fractions of 3 ml were collected.

Hydroxylapatite chromatography at pH 7.4 : The pooled fractions (30 ml) were concentrated by ultrafiltration (Amicon PM-10) and dialyzed against 25 mmol/l potassium phosphate (pH 7.4), 1.5 mmol/l $MgCl_2$, 15 % glycerol. The dialyzed fraction was applied to a column (1.6 x 5 cm) of hydroxylapatite (HA-Ultrogel, IBF, Clichy, France), equilibrated with 25 mmol/l potassium phosphate (pH 7.4), 1.5 mmol/l $MgCl_2$, 15 % glycerol. Elution was performed with a linear gradient (2 x 60 ml) from 25 mmol/l to 400 mmol/l potassium phosphate (pH 7.4) at a flow rate of 12 ml/h. Fractions of 1 ml were collected.

Hydroxylapatite chromatography at pH 8 : The pooled active fractions (10 ml) were dialyzed against 25 mmol/l potassium phosphate (pH 8), 1.5 mmol/l $MgCl_2$, 15 % glycerol and then applied to a column (1 x 3 cm) of hydroxylapatite previously equilibrated with the same buffer. Elution was performed with a double linear gradient (2 x 20 ml) from 25 mmol/l potassium phosphate (pH 8) to 400 mmol/l potassium phosphate (pH 7.4) at a flow rate of 9 ml/h. Fractions of 1 ml were collected.

First AH-Sepharose 4B chromatography : To the pooled active fractions (7 ml) ammonium sulfate was added to 2.3 mol/l at 4°C. After standing for 60 min and adjustment to pH 7.2 with KOH, the solution was applied to a column (1.6 x 7 cm) of AH-Sepharose 4B (Pharmacia, Uppsala) and equilibrated with 20 mmol/l potassium phosphate (pH 7.2), 10 % glycerol and 2.3 mol/l ammonium sulfate. Elution was carried out with a gradient (2 x 70 ml) from 2.30 to

0.42 mol/l ammonium sulfate in the same buffer. The flow rate was 7 ml/h. Fractions of 2 ml were collected.

Second AH-Sepharose 4B chromatography : The pooled active fractions (10 ml) were concentrated by ultrafiltration (Amicon PM-10) and dialyzed against 20 mmol/l potassium phosphate (pH 7.4), 1.5 mmol/l $MgCl_2$ and 10 % glycerol. The dialyzed solution was applied to a column (1.6 x 7 cm) of AH-Sepharose 4B equilibrated and eluted with the same buffer at a flow rate of 7 ml/h. Fractions of 2 ml were collected.

The active fractions were pooled, concentrated by ultrafiltration, dialyzed against 20 mmol/l potassium phosphate (pH 7.4), 50 % glycerol and kept at - 20°C.

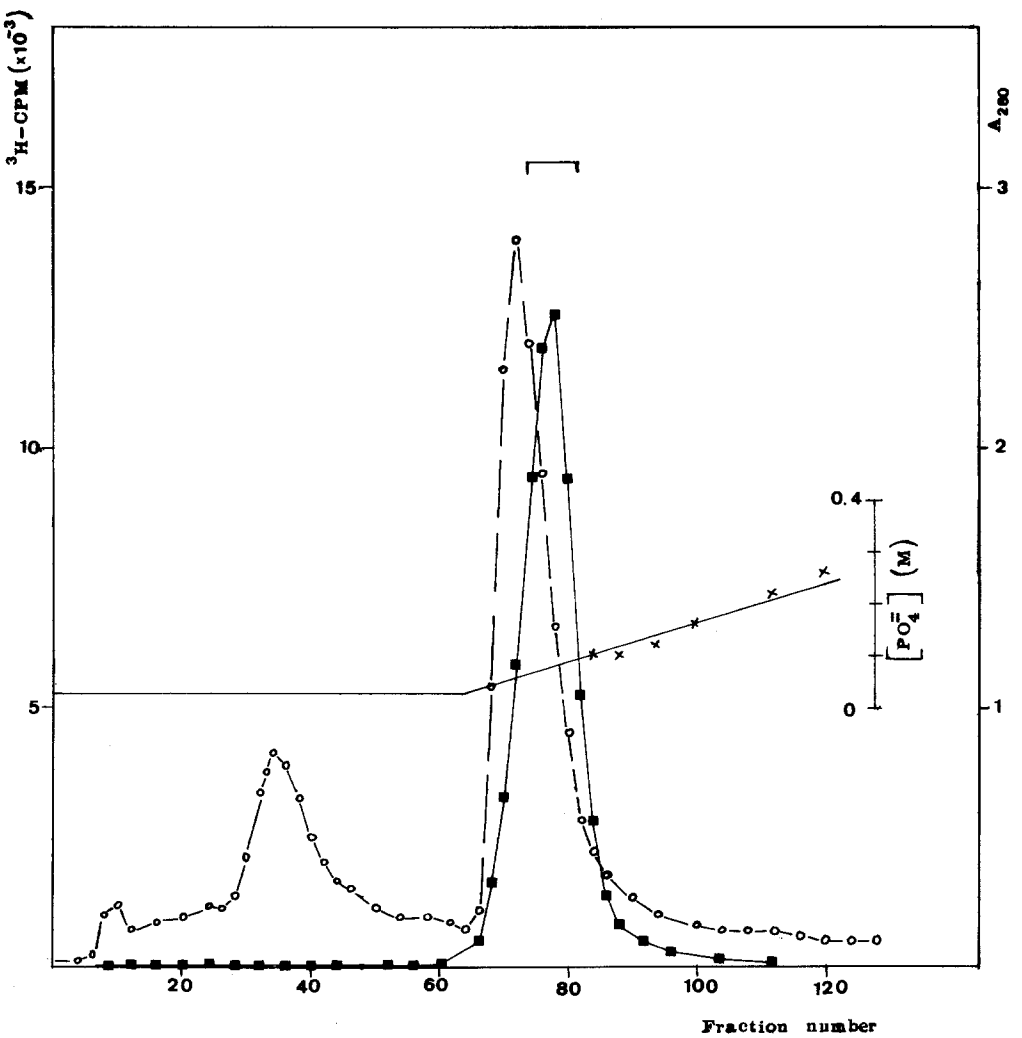

Fig. 1 : Hydroxylapatite chromatogram at pH 7.4.
O——O A_{280} nm
■——■ mitochondrial Phe-tRNA synthetase activity
X——X phosphate concentrations.

RESULTS AND DISCUSSION

Starting with 400 g of wet cells, we obtained 856 mg protein in the mitosol fraction, which represents the crude extract in table I. Precipitation with ammonium sulfate removes more than 50 % of inactive proteins and enhances the enzyme activity as the result of removal of an inhibitor. Mitochondrial Phe-tRNA synthetase activity appears in the first peak from the DEAE-cellulose column. A second peak can be eluted with 60 mmol/l KCl and contains only inactive material (not shown). With hydroxylapatite column chromatography (figure 1), the enzyme is eluted with 100 mmol/l phosphate at pH 7.4. Fraction

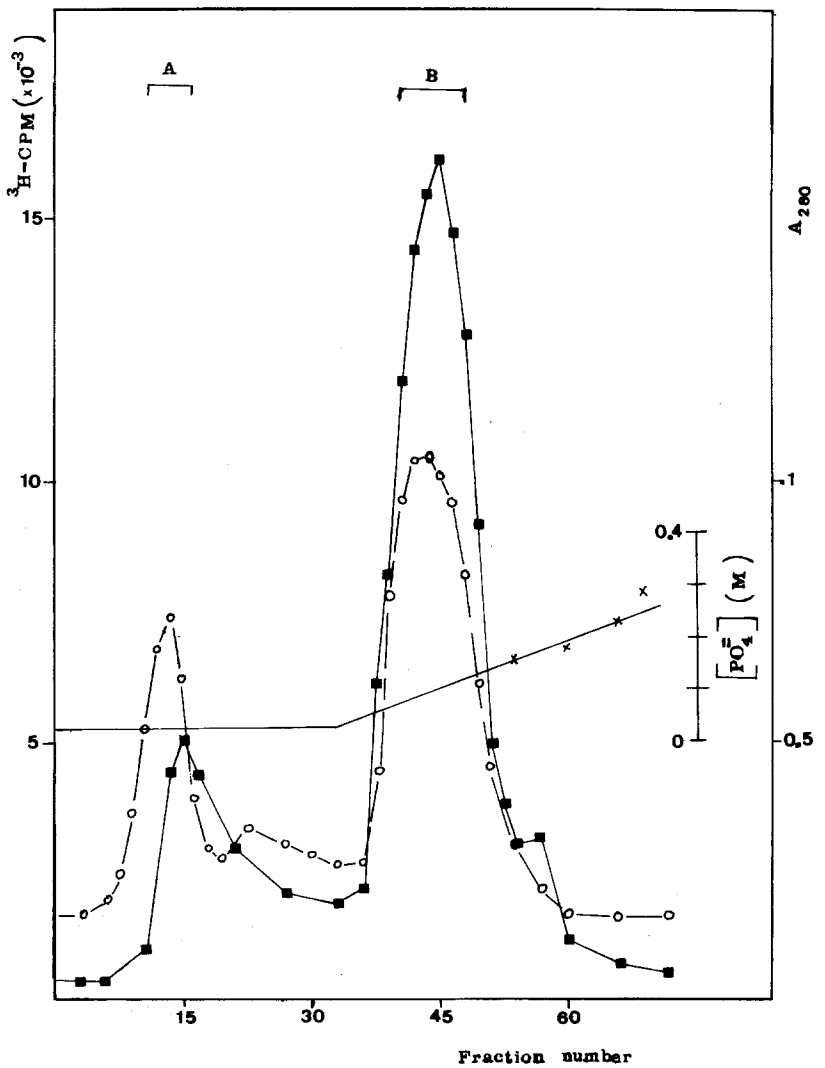

Fig. 2 : Hydroxylapatite chromatogram at pH 8.0. Legend as in figure 1

A in the second hydroxylapatite chromatogram (figure 2) does not contain a different form of the mitochondrial enzyme, but in that fraction the enzyme is non-covalently linked to some other protein. When peak A is concentrated and rechromatographed on the same column, the active fraction elutes when the phosphate concentration reaches 100 mmol/l, in the same way as for fraction B in figure 2.

Sepharose 4B chromatography with an ammonium sulfate decreasing gradient (Fig. 3) shows a difference between mitochondrial enzyme, which elutes at 0.95 mol/l ammonium sulfate (= 22 % ammonium sulfate saturation at room temperature) and cytoplasmic enzyme, which comes out at 1.83 mol/l (42 % ammonium

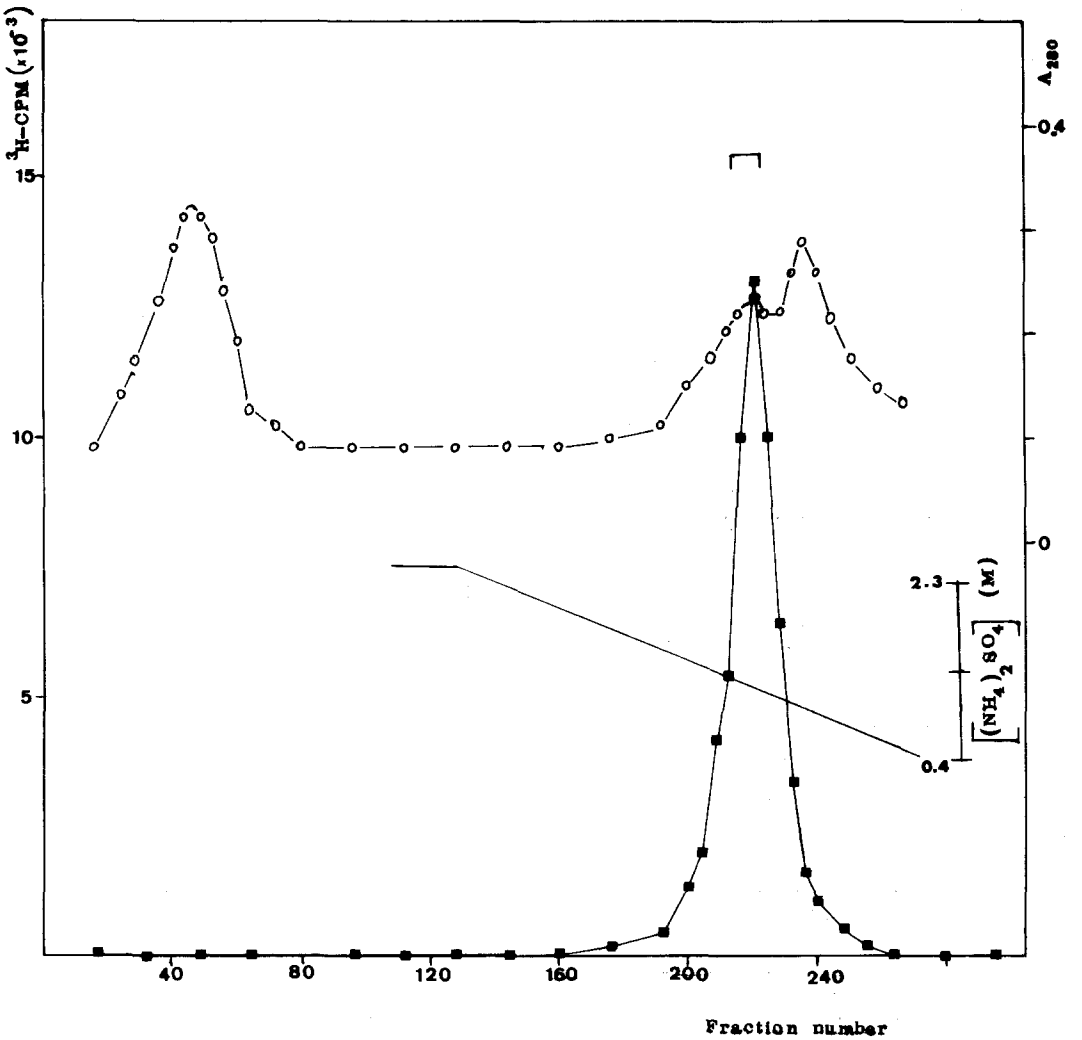

Fig. 3 : AH-Sepharose 4B chromatogram with ammonium sulfate gradient
Legend as in figure 1

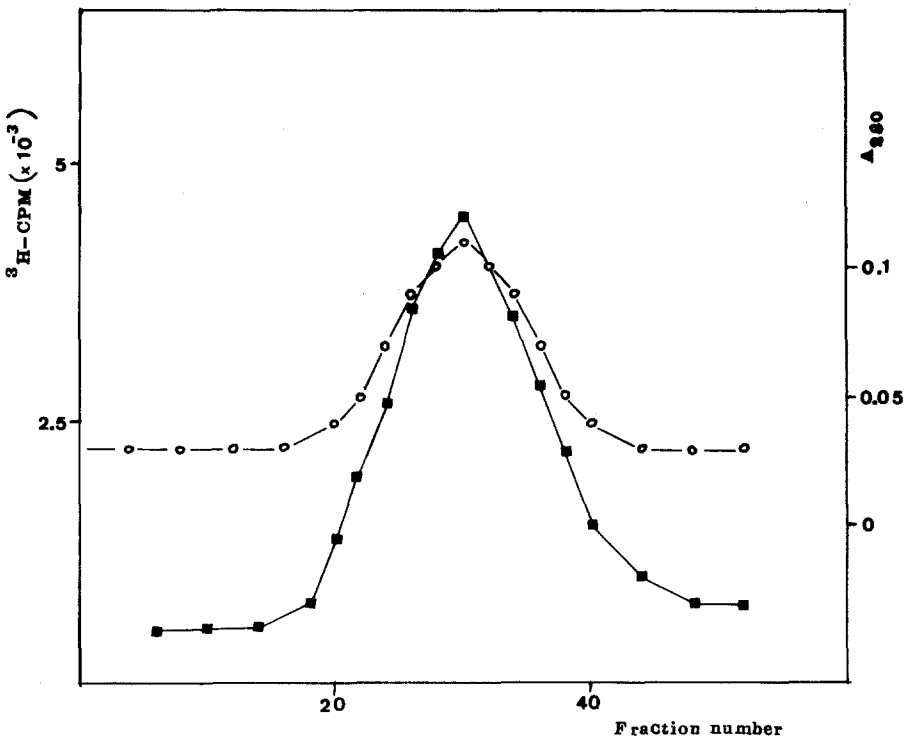

Fig. 4 : AH-Sepharose 4B chromatogram without ammonium sulfate gradient
Legend as in figure 1

TABLE I : PURIFICATION OF THE MITOCHONDRIAL Phe-tRNA SYNTHETASE

STEP	TOTAL PROTEIN (mg)	TOTAL UNITS *	SPECIFIC ACTIVITY (Units/mg protein)	YIELD (%)
Crude extract	855.60	876	1	100
Ammonium sulfate (0 - 70 %)	404.60	1229	3	140
DEAE-cellulose	106.40	791	7	90
Hydroxylapatite (pH 7.4)	15.60	552	35	63
Hydroxylapatite (pH 8)	6.44	388	60	44
AH-Sepharose 4B with $(NH_4)_2SO_4$	1.92	323	170	37
AH-Sepharose 4B	0.40	154	385	17

* The enzymatic unit is defined as 1 nmol of Phe-tRNA formed per min under the described assay conditions.

sulfate saturation at room temperature), as reported by Von der Haar (11). The second Sepharose 4B chromatography without ammonium sulfate (Fig. 4) improves the purity of the enzyme, which elutes from the column as a single peak coinciding with the 280 nm absorption. Contaminating proteins appear later during chromatography when the ionic strength is raised (not shown).

The detailed results of specific activity improvement and yields are shown in table I. The mitochondrial synthetase activity is purified 385 fold with a yield of 17 %. From 400 g of wet yeast cells we obtain 400 µg mitochondrial Phe-tRNA synthetase. Loss of enzymatic activity is observed each time the enzyme is maintained at high dilution (gel diffusion, dialysis,...) and we found it worthwhile either to shorten or omit such steps while adding fresh phenylmethylsulfonylfluoride as protease inhibitor.

The purified mitochondrial Phe-tRNA synthetase appears homogenous after the second Sepharose 4B chromatogram (Fig. 4) and following 5 % gel electrophoresis (Fig. 5), where it moves as a single band.

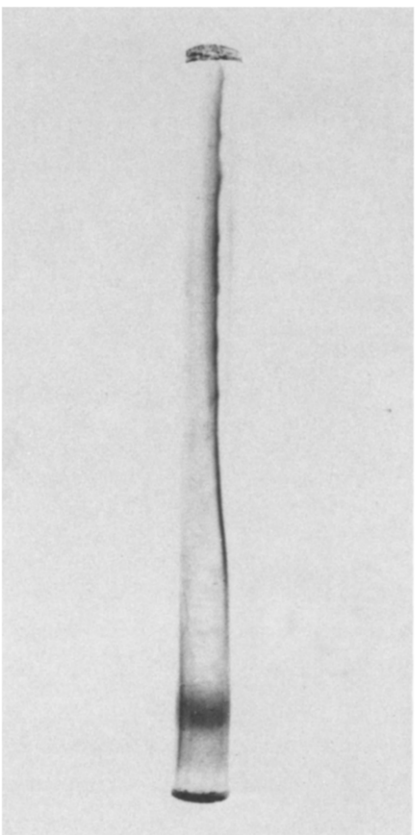

Fig. 5 : 5 % acrylamide gel electrophoresis of the purified mitochondrial Phe-tRNA synthetase

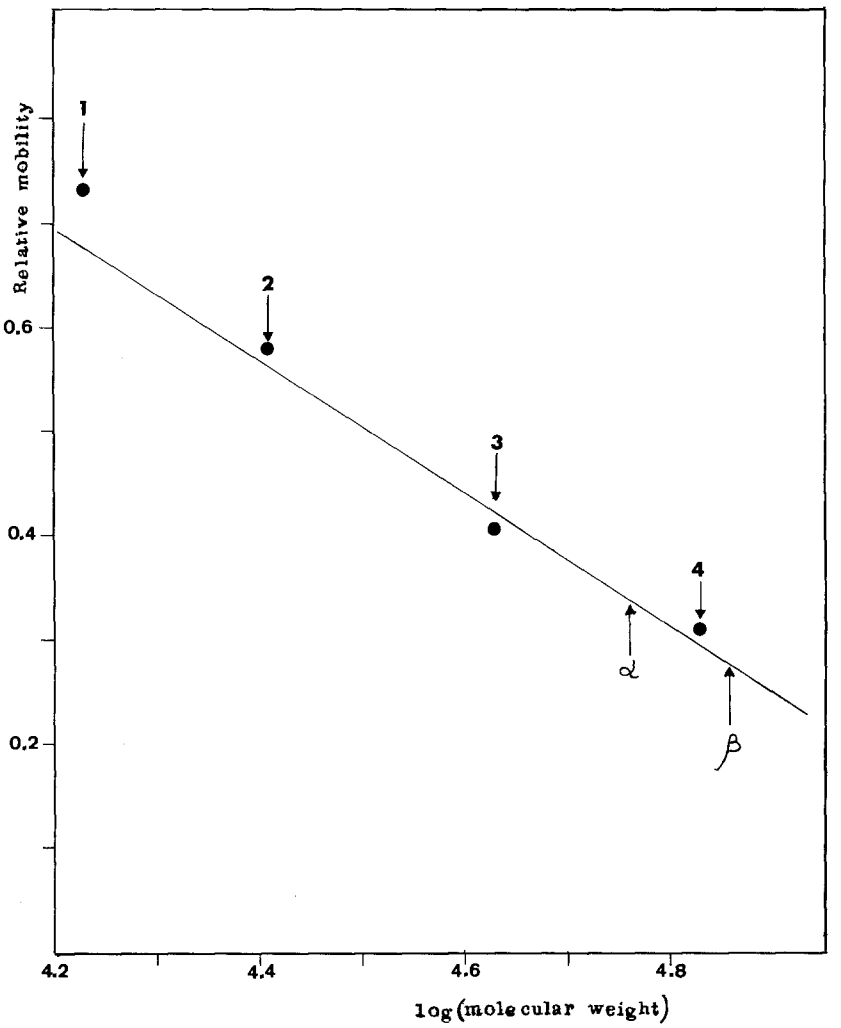

Fig. 6 : Polyacrylamide gel electrophoresis in 0.1 % SDS, 4 mol/l urea. Standards : 1 = myoglobin, 2 = chymotrypsinogen, 3 = ovalbumin, 4 = bovine serum albumin. α and β = subunits of mitochondrial Phe-tRNA synthetase.

Urea-SDS gel electrophoresis shows two bands (α and β) of molecular weights 57,000 and 72,000 respectively (Fig. 6). We note a difference in staining with Coomassie blue between weakly colored mitochondrial enzyme and very well stained cytoplasmic enzyme.

By means of analytical gel filtration (Fig. 7), the molecular weight of native mitochondrial Phe-tRNA synthetase is estimated to be 266,000. According to the molecular weight of subunits, the molecular weight of native mitochondrial Phe-tRNA synthetase should be of the order of 258,000 and the quaternary structure could therefore be written $\alpha_2 \beta_2$.

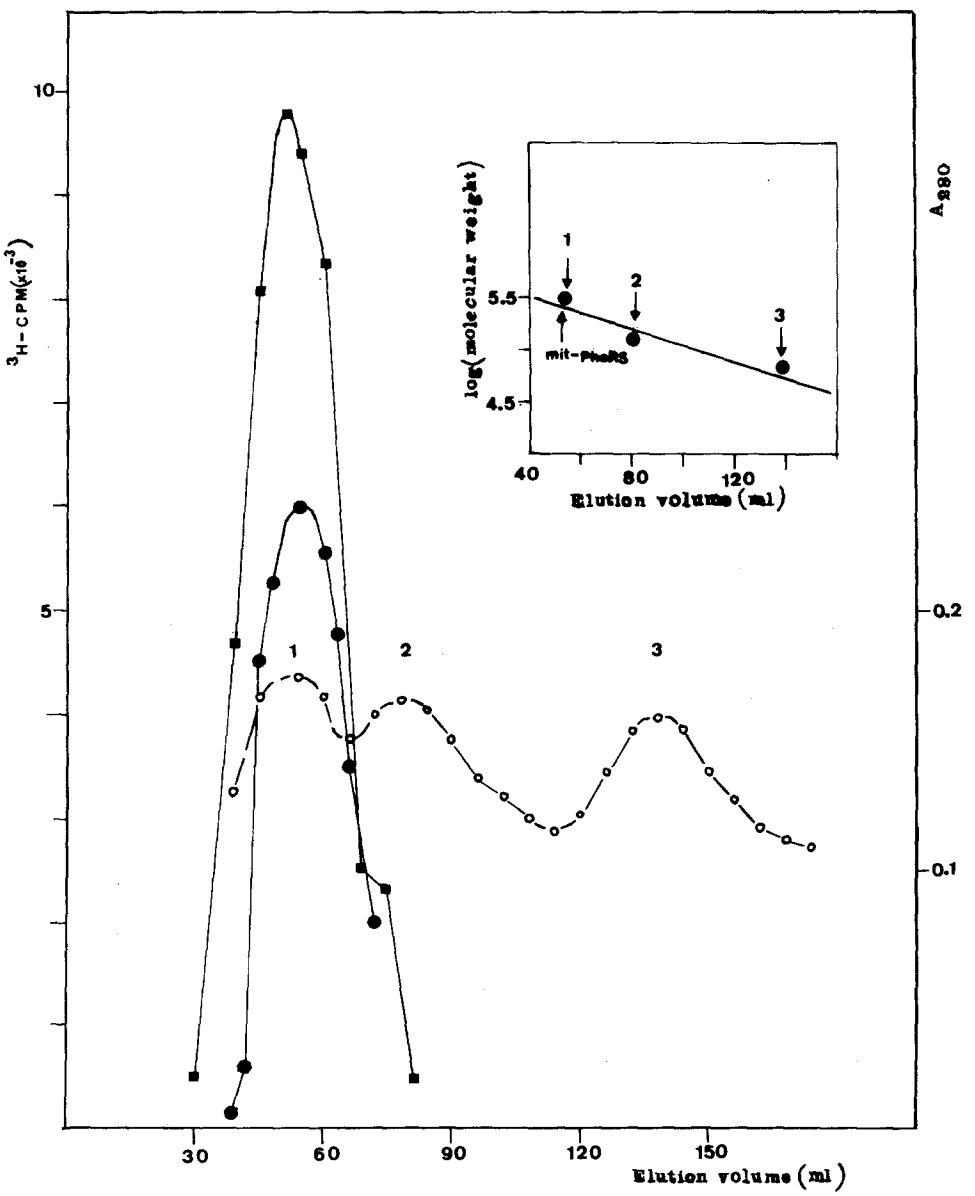

Fig. 7 : Analytical gel filtration
Standards : 1 = cytoplasmic Phe tRNA synthetase, 2 = β-galactosidase, 3 = bovine serum albumin.
O———O A_{280} nm O———O cytoplasmic Phe-tRNA synthetase activity
■———■ mitochondrial Phe-tRNA synthetase activity.

From these results, we conclude that the structure of yeast mitochondrial Phe-tRNA synthetase is analogous to that of its cytoplasmic homologous counterpart, where the same kind of structure was found earlier (8, 12). The mitochondrial enzyme resembles all Phe-tRNA synthetases already studied in various organims owing to the fact that its molecular weight is of the order of magni-

tude of 250,000 daltons and because it is comprised of four subunits, 2α and 2β. In any event, the primary structure of the mitochondrial and the cytoplasmic enzymes must be different because of their chromatographic, catalytic and immunological properties (1, 2). It is possible that the two enzymes share one of their two subunits, the other one differing with respect to the mitochondrial and the cytoplasmic enzymes. Further investigation of the primary structure of the mitochondrial Phe-tRNA synthetase is currently in progress in our laboratory.

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