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THE USE OF AFFINITY ELUTION FROM BLUE DEXTRAN SEPHAROSE BY YEAST $tRNA_2^{Val}$ IN THE COMPLETE PURIFICATION OF THE CYTOPLASMIC VALYL-tRNA SYNTHETASE FROM EUGLENA GRACILIS

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SUMMARY

Euglena gracilis cytoplasmic valyl-tRNA synthetase was purified to homogeneity. After enrichment of the enzyme by a series of classical chromatographic steps (on hydroxyapatite, phosphocellulose and DEAE-cellulose), complete purification was achieved by affinity elution from a Blue Dextran Sepharose column, using yeast $tRNA_2^{Val}$. The purified enzyme is a monomer of 126,000 daltons and has K_m values of 5×10^{-5} M for L-valine, 7×10^{-5} M for ATP and 5×10^{-8} M for Euglena $tRNA^{Val}$.

INTRODUCTION

Pionneering studies of Reger et al. (1) and Krauspe and Parthier (2) have established that the Euglena cell contains chloroplast specific aminoacyl-tRNA synthetases (E.C. 6.1.1...) which, in most cases, differ from their cytoplasmic counterpart in their chromatographic behaviour and their substrate (tRNA) specificity. In order to determine the differences between a cytoplasmic and a chloroplastic enzyme specific for the same amino acid, we decided to compare the structural and catalytic properties of the cytoplasmic valyl-tRNA synthetase and of its chloroplastic counterpart. This requires the purification of the two enzymes. In a preceding paper we have described the purification of the chloro ValRS from Euglena gracilis (3) and we are reporting here the purification of the cyto ValRS.

Affinity chromatography has been used in the purification of plant aminoacyl-tRNA synthetases and appears to be a very promising technique. Sepharose-bound tRNA has frequently been used for the purification of plant aminoacyl-tRNA synthetases with more (4) or less (5) success. Recently Drocourt et al. (6) have developed an affinity chromatography method, consisting in the specific elution of an aminoacyl-tRNA synthetase from a Blue-Dextran Sepharose column by its cognate tRNA.

Abreviations : chloro ValRS = chloroplastic valyl-tRNA synthetase ;
cyto ValRS = cytoplasmic valyl-tRNA synthetase

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We are reporting here the purification to homogeneity of Euglena cyto ValRS by affinity elution from Sepharose Blue-Dextran, using a heterologous substrate, yeast $tRNA^{Val}$, and are presenting results on its molecular weight and on some of its kinetic properties.

MATERIALS AND METHODS

Growth conditions of Euglena gracilis Z. cells, protein concentration measurements and preparation of Euglena tRNA have been previously described (3). The aminoacylation of tRNA was also performed as described (3), except that, to test the cyto ValRS, the ATP concentration was 5 mM and that yeast or Euglena tRNA was used as a substrate.
Hydroxyapatite was prepared according to Siegelman (7), DEAE-cellulose and phosphocellulose were from Whatman, Sephadex G50, G200, Sepharose 4B and Blue-Dextran were from Pharmacia. Blue-Dextran Sepharose 4B was prepared according to Ryan and Vestling (8). Yeast total tRNA was purchased from Boehringer, Mannheim. Other chemicals were from Merck.
To obtain pure $tRNA_2^{Val}$ a modification of the procedure of Bonnet et al. was used (9) : Commercial brewer's yeast tRNA was subjected to countercurrent distribution, and enriched $tRNA_2^{Val}$ fractions were chromatographed on a Sepharose 4B column (2.5 x 130 cm) in sodium acetate buffer pH 6.5, 10 mM, β-mercaptoethanol 6 mM, $MgCl_2$ 10 mM and EDTA 1 mM. Elution was performed using a reverse ammonium sulfate gradient from 2 M to 1 M in the above-mentioned buffer. The $tRNA_2^{Val}$ was further purified on a BD-cellulose column as already described (9).

Buffer A. Tris-HCl pH 8, 50 mM, $MgCl_2$ 1 mM, β-mercaptoethanol 1 mM, EDTA 0.1 mM, L-valine 2 x 10^{-3} mM, propane 1-2 diol 10% (v/v) and glycerol 10% (v/v). Buffer B. Potassium phosphate pH 7.5, 50 mM, $MgCl_2$ 1 mM, β-mercaptoethanol 1 mM, EDTA 0.1 mM, L-valine 2 x 10^{-3} mM, propane 1-2 diol 15% (v/v). Buffer C. The same as B, but 5 mM in potassium phosphate. Buffer D. The same as C but 10% (v/v) in glycerol instead of propane 1-2 diol. Buffer E. Tris-HCl pH 7.5, 20 mM, EDTA 0.1 mM, L-valine 4 x 10^{-3} mM, β-mercaptoethanol 1 mM, propane 1-2 diol 15% (v/v). Buffer F. Tris-HCl pH 7.5, 10 mM, EDTA 0.1 mM, β-mercaptoethanol 1 mM et propane 1-2 diol 15% (v/v).

RESULTS

1) Enzyme purification

Freshly harvested Euglena cells (300 g) were suspended in 300 ml of buffer A, made 2% in polyethylene glycol 6000 (w/v) and disrupted in a French press at 12,000 psi. The homogenate was centrifuged at 35,000 x g for 30 min and ammonium sulfate was added to the supernatant to 70% saturation. The resulting precipitate was redissolved in buffer B, desalted on a Sephadex G50 column (7 x 40 cm) and applied to a hydroxyapatite column (6 x 14 cm). This column, as already reported (3), separates the chloro ValRS from the cyto ValRS, so that prior purification of the chloroplasts is not necessary. As shown on fig. 1 the chloroplastic enzyme is not retained on the column, while the cyto ValRS is eluted by a linear gradient from 50 to 400 mM potassium phosphate in buffer B (2 x 2 l). The fractions containing the cyto ValRS were dialysed against buffer C and applied to a phosphocellulose column (4 x 17 cm) equilibrated with buffer C (fig. 2). After washing

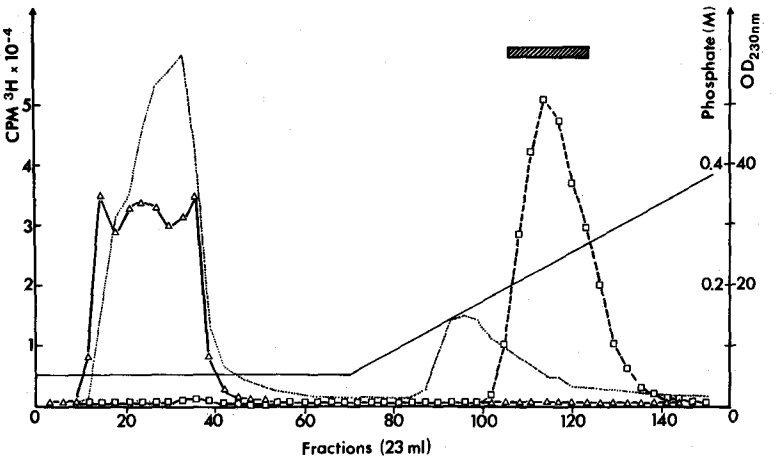

FIG. 1. Hydroxyapatite chromatography. The chloro ValRS activity was measured using E.coli tRNA (Δ—Δ) and the cyto ValRS activity using yeast tRNA (□--□) ; (••••••) $A_{230\ nm}$; (———) potassium phosphate concentration ; (▨) fractions collected and pooled for the next purification step.

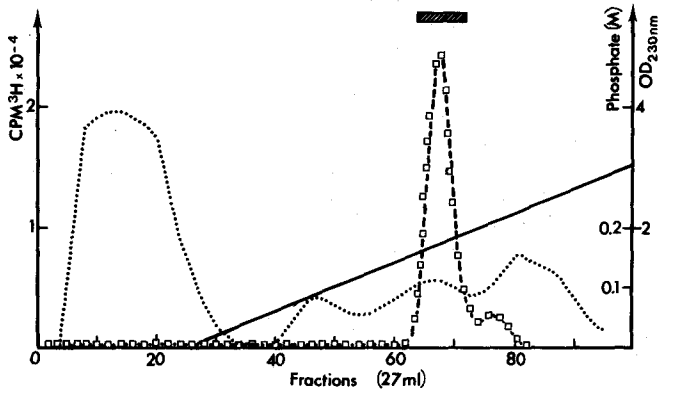

FIG. 2. Phosphocellulose chromatography. (□--□) cyto ValRS activity ; (••••••) $A_{230\ nm}$; (———) potassium phosphate concentration ; (▨) fractions pooled for the next purification step.

the column with buffer D, elution was performed with a linear gradient from 5 mM to 300 mM potassium phosphate in buffer D (2 x 1 l). Active fractions were pooled, dialysed against buffer E and loaded on a DEAE-cellulose column (3 x 14 cm) equilibrated with buffer E (fig. 3). After washing with buffer E, a linear gradient from 0 to 400 mM KCl in buffer E was applied. The fractions containing the ValRS activity were pooled, dialysed for 4 h against buffer F and applied to a Blue-Dextran Sepharose column (3 x 14 cm) previously equilibrated with buffer F. After washing the column, specific elution was performed with 200 ml of buffer F containing 10^{-6} M yeast $tRNA_2^{Val}$ (purified as described in Materials and Methods).

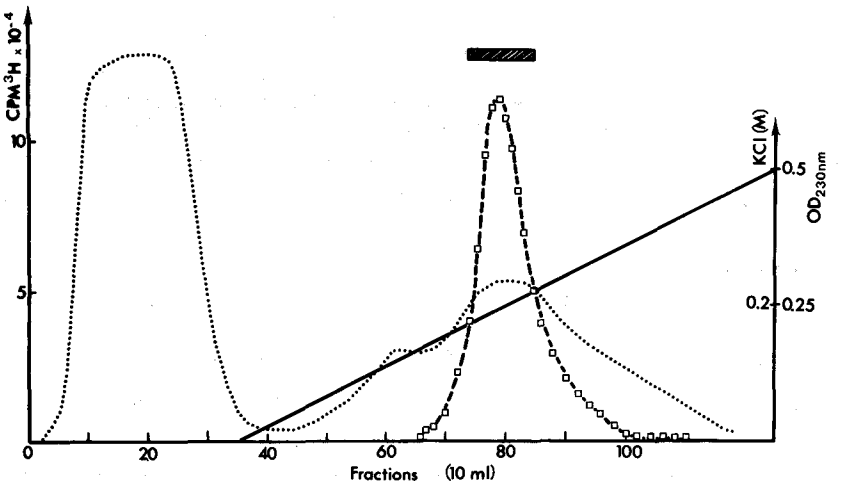

FIG. 3. DEAE-cellulose chromatography. (□--□) cyto ValRS activity ; (••••••) $A_{230\ nm}$; (———) KCl concentration ; (▨) fractions pooled for the next purification step.

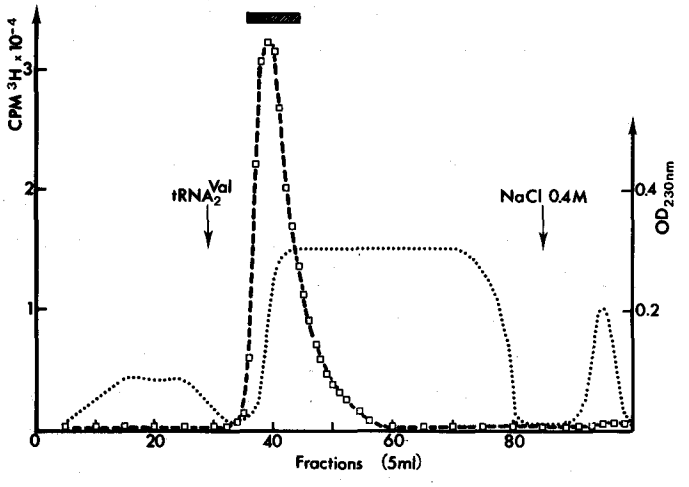

FIG. 4. Blue-Dextran Sepharose chromatography. (□--□) cyto ValRS activity ; (••••••)$A_{230\ nm}$; (▨) fractions pooled.

As shown on fig. 4, the cyto ValRS is immediately eluted when its heterologous substrate is added and only a negligible amount remains bound to the column. Fractions 36-45 containing the pure cyto ValRS were used either for electrophoretic analysis or for K_m determinations.

Whenever necessary, the $tRNA_2^{Val}$ contaminating the enzyme was separated (and recovered) on a small DEAE-cellulose column : The enzyme is eluted with 250 mM NaCl in buffer E and the $tRNA_2^{Val}$ with 1 M NaCl in buffer E.

TABLE I
Purification of cytoplasmic ValRS

	Protein (mg)	Specific activity (Units/mg)	Units*	Purification (fold)	Yield (%)
35,000 x g supernatant	9,200	0.5	4,600	1	100
Hydroxyapatite	497	5	2,485	10	54
Phosphocellulose	25.5	54	1,380	108	30
DEAE-cellulose	8.55	80	684	160	15
Blue-Dextran Sepharose	0.6	380	228	760	5

* One unit of enzyme catalyses the aminoacylation of 1 nmole of yeast tRNA in 1 min at 30°C.

The cyto ValRS obtained after the affinity chromatography is remarkably stable. It can be stored at 4°C and keeps at least 80% of its activity after one month. The purification procedure is summarized in table I.

2) Control of enzyme purity

No other aminoacyl-tRNA synthetase activity could be detected, when tested with a mixture of ^{14}C amino acids in the presence of an excess of non-radioactive L-valine. The enzyme gives a single band upon polyacrylamide gel electrophoresis under denaturing conditions (fig. 5).

3) Molecular weight and kinetic parameters of the enzyme

Under denaturing conditions (on polyacrylamide gel electrophoresis in urea-SDS) and under non-denaturing conditions (upon Sephadex G200 filtration) the cyto ValRS co-migrates with the chloro ValRS which is known to be a monomer of

FIG. 5. SDS polyacrylamide gel electrophoresis of the purified cyto ValRS. The electrophoresis was performed on 15 µg of purified enzyme (denatured by incubation at 100°C (for 4 min in the presence of 0.1% SDS, urea 4 M, β-mercaptoethanol 5%) on a 7.5% polyacrylamide gel, according to Laemli (10). After electrophoresis the gel was stained with Coomassie Brillant Blue.

TABLE II
Km values of cytoplasmic ValRS for various substrates

Substrate	K_m
L-valine	5×10^{-5} M
ATP	7×10^{-5} M
Euglena gracilis $tRNA^{Val}$	5×10^{-8} M
Yeast $tRNA_2^{Val}$	8×10^{-8} M

The K_m values for $tRNA^{Val}$ were determined by using total Euglena gracilis tRNA or purified yeast $tRNA_2^{Val}$. In the case of Euglena $tRNA^{Val}$, the K_m values were corrected to take into account the percentage of $tRNA^{Val}$ contained in the total tRNA (this percentage was determined from the aminoacylation plateau values of total tRNA with valine).

126,000 daltons (3). These results suggest that the cyto ValRS is also a monomer of 126,000 daltons.

The K_m values of the cyto ValRS have been determined for various substrates (with respect to the aminoacylation reaction) and are summarized in table II.

DISCUSSION

Throughout this work, for routine tests of cyto ValRS activity, commercial yeast tRNA (Boehringer, Mannheim) was used, which is as good a substrate as Euglena tRNA, as judged from their almost identical K_m values (table II).

Cyto ValRS is an extremely sensitive enzyme as already observed by Krauspe and Parthier (2), even after partial purification on hydroxyapatite. It is in fact very difficult to maintain the activity, especially during the early stages of the purification procedure. The addition to the buffers of protease inhibitors, such as phenyl methyl sulphonyl fluoride (PMSF) or diisopropylfluorophosphate (DIFP), did not result in any protection of the enzyme. The problem of enzyme degradation was overcome essentially by using the appropriate buffers (containing L-valine) and by performing the purification rapidly (especially the first steps).

The crucial step in the purification procedure, which enabled us to purify the cyto ValRS to homogeneity is the affinity chromatography. In the purification of the chloro ValRS from Euglena gracilis, we used Blue-Dextran Sepharose chromatography and eluted the enzyme with ATP and L-valine (3). But this method was unsuccessful in the case of the cyto ValRS. As already reported for the tryptophanyl-tRNA synthetase from E.coli (6), it seems that aminoacyl-tRNA synthetases

can form a complex with Blue-Dextran Sepharose via their tRNA binding site rather than their ATP binding site. We therefore performed the elution from Blue-Dextran Sepharose using a heterologous substrate of cyto ValRS, namely yeast $tRNA_2^{Val}$ purified from commercially available yeast total tRNA. This elution is highly specific, as suggested by the fact that before this affinity step the enzyme is contaminated by other synthetases (revealed by a test with a mixture of 15 ^{14}C-amino acids in the presence of an excess of non-radioactive L-valine), while after this step no other synthetase could be detected.

The DEAE-cellulose chromatography appeared necessary in order to ensure that endogenous tRNA, which could be attached to the cyto ValRS, was completely eliminated and would not interfere with the affinity chromatography (which is the next step).

The K_m values obtained for the various substrates of cyto ValRS are similar to those found for ValRS from other sources (3, 11-13).

Cyto ValRS appears to be a monomer of 126,000 daltons, a value similar to that obtained for its chloroplastic counterpart (3) and for the ValRS of other species (11-13).

Experiments are now in progress in our laboratory to compare the catalytic and structural features of Euglena chloro and cyto ValRS.

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