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PURIFICATION AND PROPERTIES OF VALYL-tRNA SYNTHETASE FROM *MYCOBACTERIUM SMEGMATIS*

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Summary

Valyl-tRNA synthetase from *Mycobacterium smegmatis* has been purified over 1200-fold by conventional techniques as well as affinity chromatography on valyl-aminohexyl Sepharose columns. The purified preparation is homogeneous by electrophoretic and immunologic criteria. The enzyme is a tetramer of approximate molecular weight of 120 000, composed of a single type of subunit. The synthetase exhibited maximal activity between 35—40°C and pH 6.8—7.0. The pure enzyme though stable for several months below 0°C, loses activity completely at 70°C, for 1 min. The enzyme showed normal Michaelis-Menten kinetic behaviour in the total aminoacylation reaction with K_m values of 1.25 μM, 0.1 mM and 1.0 μM for valine, ATP and tRNA, respectively, but the kinetic response deviated from the above pattern in the partial (activation) reaction. Based on these findings, the existence of the enzyme in two molecular forms, modulated by substrate concentration has been suggested; of these, only one may be active in the total reaction, while both forms may function in the prophosphate exchange reaction.

Introduction

Aminoacyl-tRNA synthetases have been purified from a variety of eukaryotic and prokaryotic cells [1—4]. Amongst the prokaryotic sources, the investigations have mostly been confined to *Escherichia coli* or some *Bacillus* species. In general, the synthetases vary in size from 45 000 to 200 000 daltons and they are composed of one or more identical subunits, of the type α, α_2 or α_4 or rarely of the type $\alpha_2\beta_2$.

In almost all the cases examined, a single enzyme (aminoacyl-tRNA synthetase) recognises the different species of a cognate tRNA, specific for that amino acid. In the course of our investigations on aminoacyl-tRNA synthetases from Mycobacteria, however, we have shown that methionyl-tRNA synthetase from *M. smegmatic* exists in two forms A and B, one of which is specific for the initiator species $tRNA_f^{met}$ [5]. The presence of more than one Met-tRNA synthetase from wheat germ has also been subsequently reported [6].

We, therefore, extended our investigations to the other aminoacyl-tRNA synthetases from *M. smegmatis* but did not find any heterogeneity in any of the synthetases tested. In the present paper, we report the purification of valyl-tRNA synthetase from this organism, using conventional and affinity chromatographic techniques. The properties and abnormal kinetic behaviour of the enzyme are also presented.

Materials and Methods

L-[U-^{14}C]Valine and [^{32}P]pyrophosphate were from Bhabha Atomic Research Centre, Bombay, India. Aquacide-I was from Calbiochem, La Jolla, CA, U.S.A. All of the other biochemicals, enzymes used as molecular weight markers and reagents for polyacrylamide gel electrophoresis, were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Buffer A : 10 mM Tris (pH 7.6)/20 mM β-mercaptoethanol/5 mM $MgCl_2$ and 10% glycerol.

Buffer B : 20 mM potassium phosphate (pH 6.7)/20 mM β-mercaptoethanol/5 mM $MgCl_2$ and 10% glycerol.

Organisms. M. smegmatis SN2 was grown on a synthetic liquid medium [7] in presence of 0.1% Tween-80, at 37° C and with aeration. Cells were harvested during late exponential phase (36 h) and stored at —20° C till use.

Enzyme assays. The enzyme assays were carried out as the amino acid acceptor assay (total reaction) or the pyrophosphate exchange assay (partial reaction).

The standard amino acid acceptor assay system contained in a final volume of 0.125 ml/100 mM Tris (pH 7.0)/10 mM $MgCl_2$/10 mM KCl/2 mM ATP/50 μg of total tRNA/1.5 μM, [^{14}C]valine and the enzyme. After incubation at 37° C for 15 min, 0.1-ml samples were removed on to Whatman 3 MM filter paper square (1 cm^2) and the acid precipitable radioactivity was determined using a Beckman Model LS 100 scintillation spectrometer. One unit of enzyme activity is defined as the amount of enzyme that incorporates 1 nmol of [^{14}C]-valine into tRNA in 15 min at 37° C under the above conditions.

The ATP-[^{32}P]pyrophosphate exchange reaction was carried out according to the method of Calender and Berg [8]. The standard assay system contained : 100 mM Tris (pH 7.0)/10 mM $MgCl_2$/10 mM KCl/2 mM ATP/2 mM valine/1.5—2.0 mM [^{32}P]$Na_2P_2O_7$. At the end of incubation, the [^{32}P]ATP was determined, after adsorption on to charcoal.

The unit of enzyme activity in this reaction is defined as the amount that catalyzes the formation of 1 nmol of ATP under the experimental conditions.

Protein estimations were carried out by the method of Lowry et al. [9].

The total tRNA from *M. smegmatis* was isolated and passed through DEAE cellulose, as described earlier [10,11].

Polyacrylamide gel electrophoresis at pH 8.6 and pH 4.3 were carried out by the methods of Ornstein [12] and Davis [13]. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was run according to the method of Weber and Osborn [14]. The molecular weight determination by the electrophoretic method was carried out according to Hedrick et al. [15].

Isolation and purification of enzyme. The frozen cells were suspended in buffer A (2 mg/g) and disrupted in a French Pressure Cell (Amincon) at 18 000 psi. The S100 supernatant was prepared by centrifugation of the clarified extract at 100 000 × *g* for 90 min. To free the enzyme from endogenous tRNA, the S100 was passed through a DEAE cellulose column equilibrated with buffer A and eluted using buffer B containing 0.4 M NaCl. The protein fractions were pooled and precipitated with ammonium sulphate; the fraction precipitating between 30 and 75% of ammonium sulphate was dissolved in buffer B and dialysed against the same buffer. This fraction was chromatographed on a column of DEAE cellulose and the elutions were carried out using a double gradient of potassium phosphate and KCl (Fig. 1). The maximally active fractions were pooled, concentrated by ammonium sulphate precipitation or by dialysis against 7% Aquacide-I, and gel filtered on a column of Sephadex G-200. The active fractions from the above step were pooled and fractionated on a hydroxyapatite column (Fig. 2) using a potassium phosphate gradient (20—200 mM). The peak fractions showing enzyme activity were pooled and refiltered on a Sephadex G-200 column which resulted in a pure preparation of the enzyme.

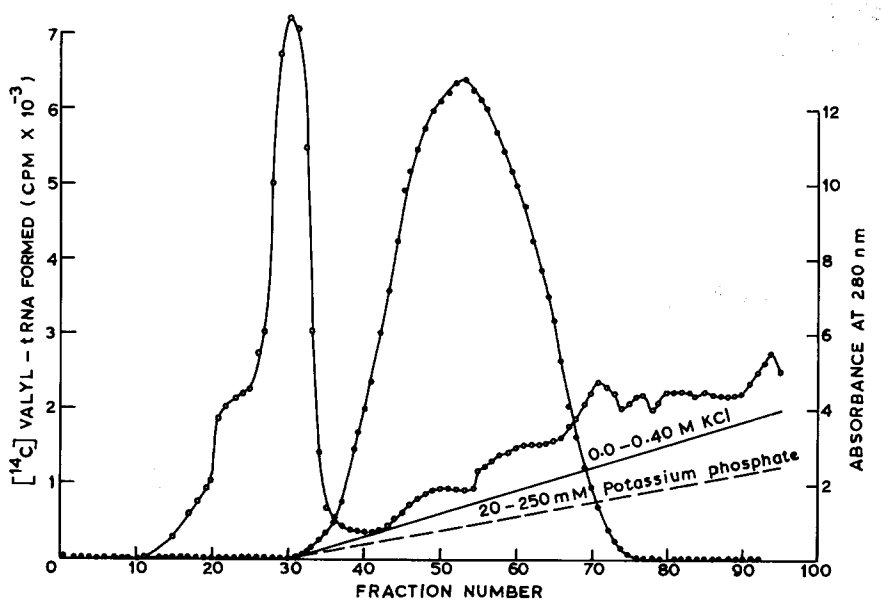

Fig. 1. Chromatography on DEAE-cellulose. The protein fraction precipitating between 30—75% saturation of ammonium sulphate (step 3) was dialysed against buffer B. This fraction was loaded on a DEAE-cellulose column (1.4 × 40 cm), equilibrated with the same buffer. Enzyme was eluted using a double gradient of KCl (0.0—400 mM) and potassium phosphate (20—250 mM). Fraction size, 2.0 ml; 10 µl samples were used for enzyme assay. ○———○, absorbance at 280 nM; ●———●, enzyme activity.

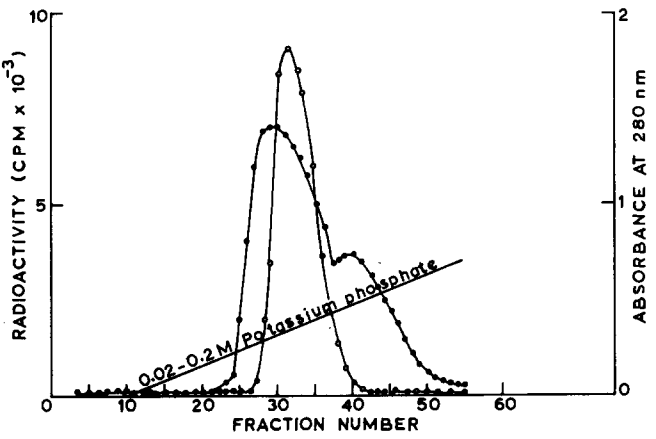

Fig. 2. Chromatography of the enzyme on hydroxyapatite. The enzyme after gel filtration on Sephadex G-200 (step 5) was loaded on to a hydroxyapatite column (1.0 X 20 cm), equilibrated with buffer B. Enzyme was eluted using a linear gradient of potassium phosphate (20—200 mM). Fraction size, 1.4 ml; 10 μl samples were used for enzyme assay ●———●, absorbance at 280 nM; ○———○, Enzyme activity.

Enzyme purification by affinity chromatography: (i) preparation of affinity column. Valylaminohexyl Sepharose 4B was prepared by modifying the method of Robert-Gero and Waller [16] used for methionyl aminohexyl Sepharose 4B synthesis. *p*-Nitrophenyl sulphenyl valine was coupled to AH-Sepharose 4B using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide as the coupling agent; the nitrophenyl sulphenyl group was subsequently removed by treatment with sodium thiocyanate.

(ii) Chromatography of the enzyme. The enzyme preparation at the stage of first Sephadex-G200 filtration (Step 5, Table I) was loaded on to a column of valyl aminohexyl Sepharose 4B (0.5 X 3 cm), previously equilibrated with

TABLE I

PURIFICATION OF VALYL-tRNA SYNTHETASE FROM *MYCOBACTERIUM SMEGMATIS*

Fractionation step	Total protein (mg)	Specific * activity	Fold purification	Yield (%)
1 S100	1125	0.049	1	
2 DEAE-Cellulose-I chromatography	384	1.476	30.12	100 **
3 30—75% Ammonium sulphate fraction	108	3.6	75.0	69
4 DEAE-cellulose-II chromatography	25.6	7.4	148.0	33
5 Sephadex G-200 gel filtration	8.06	17.5	357.0	25
6 Hydroxyapatite chromatography	4.97	27.6	563.0	24
7 Refiltration on Sephadex G-200	0.4	59.5	1214.0	4.2
6a Affinity chromatography on valyl-aminohexyl Sepharose ***	0.7	63.0	1285.0	7.8

* Specific activity = U/mg protein.

** The total activity at this stage was considerably more than the S100 stage, probably due to the removal of inhibitory materials and was taken as 100%.

*** The active fractions at step 5 were pooled and directly chromatographed on valyl-aminohexyl Sepharose, omitting steps 6 and 7.

buffer B, containing 0.1 M KCl. The column was washed with the same buffer and the enzyme was eluted in presence of 0.2 M KCl.

Antibody to the enzyme was raised against the protein fraction after the first gel filtration (Step 5, Table I). 1 mg of protein was injected subcutaneously to the rabbit, in Freunds adjuvant, four times at 10 day intervals, the animal was bled and the antiserum was collected. Immunodiffusion was carried out by the Ouchterlony double diffusion technique.

Results

The summary of purification of valyl-tRNA synthetase from *M. smegmatis* is given in Table I.

The purification steps resulted in a homogenous enzyme preparation with a yield of 4—8% and 1200-fold increase in specific activity.

At step 7 (refiltration on Sephadex G-200 column) during purification, the individual fractions showing high enzyme activity were subjected to electrophoresis on polyacrylamide gels. Only those fractions showing a single protein band on electrophoresis were pooled at this stage. The specific enzyme activity of the pooled fractions as well as the electrophoretic homogeneity were then determined. The enzyme showed single band on polyacrylamide gel electrophoresis at pH 4.3 and 8.6.

The enzyme was purified to a homogenous state by the affinity chromatographic technique, after the first Sephadex gel filtration, as an alternative procedure. The specific activity of this enzyme preparation was very similar to that of the other method, but the yield was better.

The pure enzyme was also antigenically homogeneous, as shown by the single immunodiffusion band (Fig. 3).

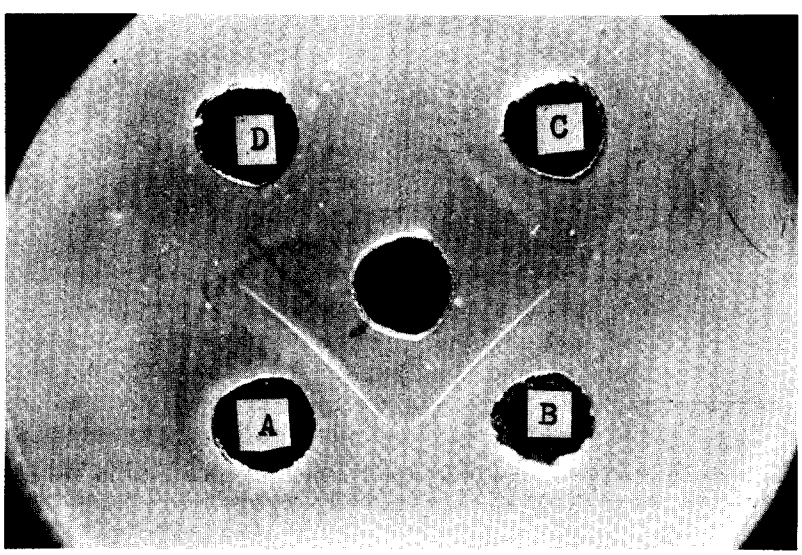

Fig. 3. Immunodiffusion pattern. The central well contained the pure enzyme, A, B, C, various concentrations of antiserum (original, 1 : 1, 1 : 10 dilutions). D control serum.

The molecular weight of the enzyme was found to be 126 000, as determined by gel filtration on a calibrated column of Sephadex G-200, and 118 000 by the electrophoretic method. SDS-polyacrylamide gel electrophoresis of the enzyme showed a single species of protein of molecular weight 30 000 suggesting that the holoenzyme may be a tetramer.

Properties of the enzyme

Valyl-tRNA synthetase from *M. smegmatis* utilised total tRNA from *E. coli* or *M. smegmatis* to the same extent under all conditions tested. The enzyme showed maximum activity between 35—40°C and pH between 6.8 to 7.0 in a number of buffer systems. The activities in the ionic buffers like piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) or *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) were better than in Tris (Fig. 4).

The optimum Mg^{2+} concentration required was 5 mM in the total reaction and 10 mM in the partial reaction (Fig. 5). Other divalent cations like Mn^{2+} and Ca^{2+} showed 60 and 16% activity at 5 mM concentrations.

Effect of substrate analogues and inhibitors

dATP has been found to be active in all the aminoacyl-tRNA synthetases tested so far [17]; in contrast the valyl-tRNA synthetase from *M. smegmatis* did not recognise dATP as a substrate. Other nucleotide triphosphates such as GTP, CTP, UTP or dGTP and dTTP were also inactive. Analogues of valine like

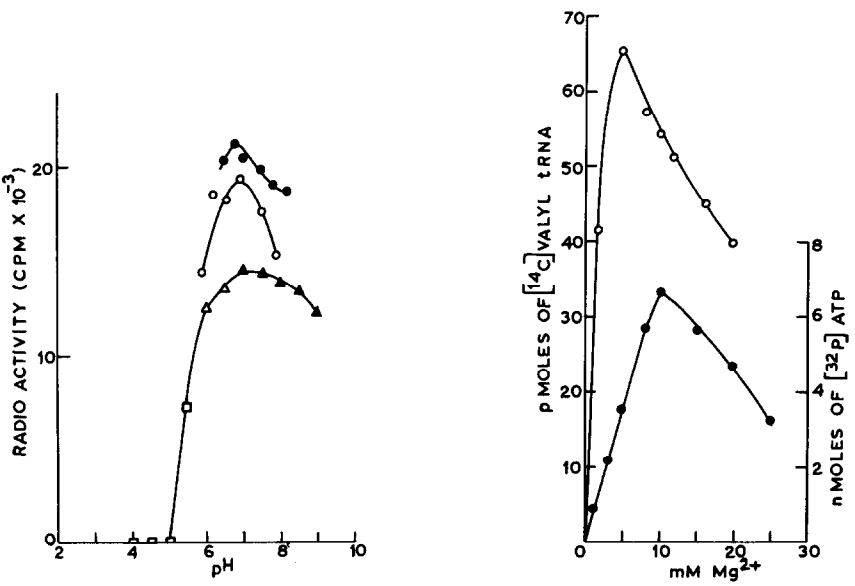

Fig. 4. Effect of pH on enzyme activity. The enzyme was assayed in different buffers using the standard assay system. ●——●, Hepes; ○——○, Pipes; □——□, sodium acetate; △——△, cacodylate; ▲——▲, Tris-HCl.

Fig. 5. Enzyme activity at different Mg^{2+} concentrations. The experimental conditions were as given in text, except that the Mg^{2+} concentration was varied as shown. ○——○, pmol [^{14}C]valyl-tRNA; ●——●, nmol [^{32}]ATP.

D- and L-penicillamine, cycloleucine and L-threonine were not activated in the pyrophosphate exchange reaction. L-penicillamine, however, acted as a competetive inhibitor in aminoacylation, with a K_i of 1.6 mM.

The enzyme was sensitive to inhibition by *p*-hydroxy mecuribenzoate (100% inhibition at 0.2 mM), but was insensitive to *N*-ethylmaleimide or iodoacetamide; chelating agents like α, α-dipyridyl, diethyl dithiocarbamate and *o*-phenanthroline were not inhibitory (up to 1 mM).

Kinetic properties

When the enzyme was assayed in the amino acid acceptor assay (total reaction), a Michaelis-Menten Kinetic behaviour was observed for all the 3 substrates. The K_m values calculated for valine, ATP and tRNA are, respectively, 1.25 μM, 0.1 mM, and 1.0 μM.

However, when the enzyme was assayed in the partial reaction (PP_i exchange assay), abnormal kinetic behaviour deviating from the Michaelis-Menten pattern was observed. The partial reaction was independent of added tRNA and the kinetic behaviour was identical in presence or absence of tRNA. The effects of varying amino acid and ATP concentrations are presented in Fig. 6 and 7. The points presented in figures are still in the linear time period of the reaction. In both instances, after the initial linear response to the varying substrate concentration, there was a plateau region followed by another increase in the activity. Consequently the Lineweaver-Burk plots were non-linear and two apparent K_m values were obtained (0.35 mM 0.83 mM for valine, and 0.18 mM and 2.5 mM for ATP, respectively). This kind of response to added substrate indicated a possible negative co-operativity in the interactions of enzyme and substrate, and hence the values were also analysed by the Hill plots. The results are presented in Fig. 8A and B. It is clear from the Fig. 8A and B that the two slopes (Hill coefficient $n = 1$) were separated by a flat region, corresponding to minimal binding sites available in that region.

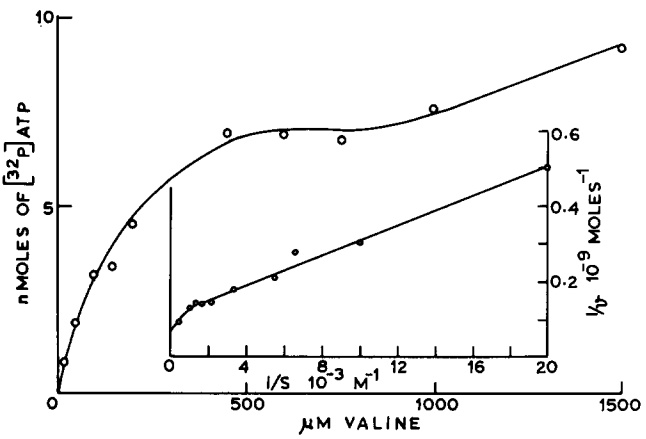

Fig. 6. Effect of valine concentration on enzyme activity in partial reaction. Assays were done as given in the text except that the valine concentration was changed from 0.0—2.0 mM. Assays were done in duplicate at two time intervals. The values shown are in the linear range of the reaction time. The inset is the Lineweaver-Burk plot of the same data.

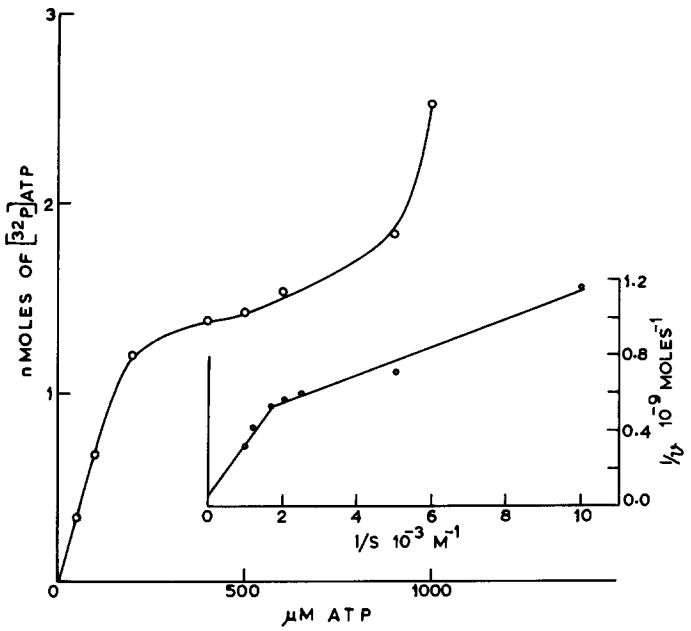

Fig. 7. Effect of ATP concentration on enzyme activity in partial reaction. Assays were done as given in the text except the ATP concentration was changed from 0.0—2.0 mM. Assays were done in duplicate at two time intervals. The inset is the Lineweaver-Burk plot of the same data.

Stability

The enzyme was stable to storage below 0° C for at least 3 months. At higher temperatures, the activity was lost rapidly. The enzyme, though stable at 60° C for 1 min, was completely inactivated at 70° C, in 1 min.

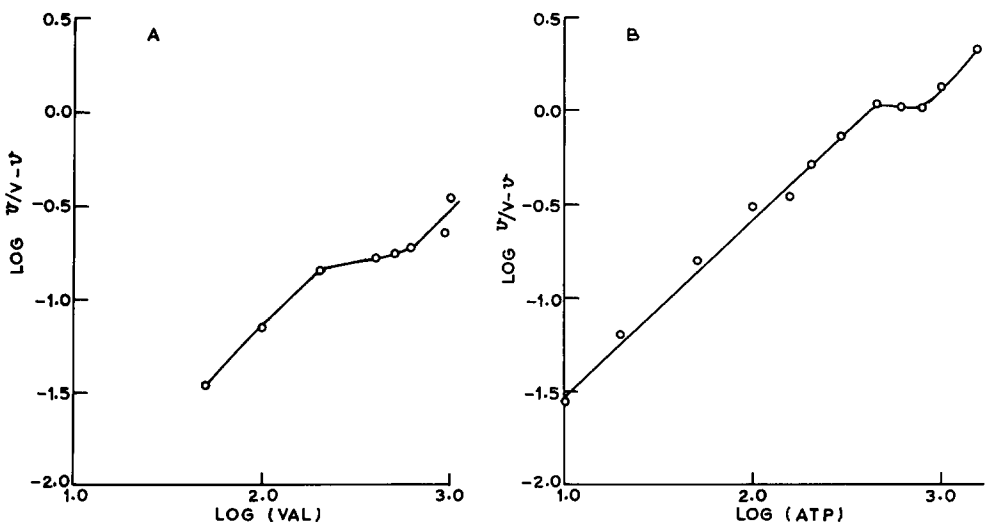

Fig. 8. Hill plots. A, Hill plot for valine in partial reaction; B, Hill plot for ATP in partial reaction. *V* values were taken from the Lineweaver-Burk plots.

Discussion

Valyl-tRNA synthetase, purified to homogeneity from *M. smegmatis*, is a tetramer with a molecular weight of 120 000. Unlike the met-tRNA synthetase from this organism, which was found to be existing in two different forms A and B, and possessing differential recognition patterns for the initiator and internal met-tRNAs [5], the valyl-tRNA synthetase did not show any heterogeneity. The general properties of the enzyme, though were similar to the other valyl-tRNA synthetases, showed significant differences in some characters. A comparison of the properties of all the valyl-tRNA synthetases in the literature is presented in Table II.

It is clear that while all the valyl-tRNA synthetases reported so far are monomeric molecules, the present enzyme is a tetramer, composed of a single type of subunit of molecular weight 30 000. The enzyme also differs from the other synthetases in molecular activity having much lower turnover numbers; this may be significant and may reflect on a general inefficiency of all the components of the protein-synthetic machinery in *M. smegmatis*, considering the relatively slow-growing nature of the organism (doubling time 150—180 min). The turnover numbers for the enzyme from *M. tuberculosis* (a slower growing organism with doubling time about 18—24 h is still lower (Natarajan, V. and Gopinathan, K.P., unpublished observations).

The most pronounced deviation of valyl-tRNA synthetase from *M. smegmatis* was in the kinetic behaviour. While the total reaction (amino acid acceptor assays) showed normal Michaelis-Menten behaviour, in the partial reaction the responses to added substrate concentrations (both amino acid and ATP), deviated substantially from that pattern. The range of valine concentration employed in the partial reaction, however, was much larger compared to the total reaction. Earlier investigations of Koshland [18,19] have revealed that kinetics of binding curves with pronounced intermediary plateau regions (e.g. those observed in the case of CTP synthetase, phosphoenolpyruvate carboxylase etc., which resemble the pattern reported in this paper) would be produced when the enzyme possessed more than two substrate binding sites and the relative magnitude of intrinsic catalytic or binding constants of these

TABLE II

COMPARISON OF VALYL-tRNA SYNTHETASE FROM DIFFERENT SOURCES

Source of enzyme	Mol.wt.	Subunit structure	Molecular activity *		Reference
			PP_i exchange reaction	Total reaction	
E. coli	110 000	α	2400	358	[17]
S. typhimurium	110 000	α			[4]
B. stearothermophilus	122 000	α			[4]
S. cerevisiae	140 000	α	3300	40	[17]
M. smegmatis	120 000	α_4	120	0.6	

* Molecular activity is defined as number of molecules of [^{32}P]ATP or aminoacyl-tRNA formed per min per mol of enzyme.

sites first decrease and then increase as the enzyme gets saturated. Such analysis of our data showed that valyl-tRNA synthetase may be regulated by negatively co-operative interactions. This type of nonlinear double reciprocal plots may also arise due to (a) the polynomial nature of the rate equation with differences in rate constants [20], and (b) existence of multiple conformational forms. Steady state kinetic data alone is not sufficient to distinguish between these mechanisms.

The phenylalanyl-tRNA synthetase from yeast (a tetramer of the type $\alpha_2\beta_2$) shows a similar response to varying substrate concentrations (to ATP and phenylalanine but not tRNA) in the total reaction [21]. This was attributed to the presence of 2 mutually interacting binding sites for the substrates, of which one or both may be operative depending on the substrate concentration. However, in subsequent investigations, Von der Haar [22] has shown that abnormal kinetic behaviour of yeast phenyl alanyl-tRNA synthetase was determined by the ionic strength of the medium. At high concentration of $MgCl_2$ and KCl, the kinetic patterns were different and the latter author has, therefore, suggested the occurrence of two different molecular species of the enzyme, which are interconvertible. Although this fact has not been unequivocally established, Von der Haar [22] based on the evidences available, has preferred it to the earlier explanation that the enzyme may possess two unequal binding sites. Under these circumstances, the valyl-tRNA synthetase from *M. smegmatis* also may be considered to be existing in 2 different molecular forms, but in this instance the interconversion between the two forms may be modulated by the substrates valine or ATP. Further, both these forms may be active in the pyrophosphate exchange reaction while only one of them can participate in the total reaction.

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