A Comparison of Purified Valyl-Transfer Ribonucleic Acid Synthetase from *Bacillus stearothermophilus* and from *Escherichia coli*

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The purification of valyl-tRNA synthetase from *Bacillus stearothermophilus* is described. The protein was greater than 90% homogeneous on polyacrylamide-gel electrophoresis after more than 850-fold purification. It has a molecular weight of 110000, and no evidence was found for the presence of subunit structure. The properties of the purified enzyme were compared with those of purified valyl-tRNA synthetase from *Escherichia coli*. The thermal stability, pH-stability and dependence of activity on the temperature and pH of the assay are reported. The two enzymes recognize and charge tRNA^{Val} from crude tRNA of the mesophile *E. coli* and of the thermophile *B. stearothermophilus*, indiscriminately. The gel-filtration method was extended to measure the binding of tRNA to synthetase directly. Binding constants for tRNA^{Val} to valyl-tRNA synthetase from *B. stearothermophilus* were determined between 5° and 60°C.

The aminoacyl-tRNA synthetases have been extensively studied in recent years, and are of special interest because, in addition to their important role in protein biosynthesis, they recognize both a small molecule, an amino acid, and a large one, tRNA, with great specificity. The instability of these enzymes has hindered both their isolation and subsequent characterization, the materials purified from such sources as *Escherichia coli* or yeast, for example, often losing activity unless stored at low temperatures in the presence of reasonable concentrations of glycerol. It was thought that the unusual thermal stability of many of the enzymes that have been isolated from *Bacillus stearothermophilus* (see, e.g., Amelunxen *et al.*, 1970; Fahey *et al.*, 1971; Muramatsu & Nosoh, 1971; Howard & Becker, 1972; Orengo & Saunders, 1972; Stenesh & Roe, 1972) indicated that this was a possible source of more stable aminoacyl-tRNA synthetases. Indeed L. Johnson & D. Söll (personal communication from Dr. D. Söll) have shown that six of the 20 aminoacyl-tRNA synthetases from *B. stearothermophilus* are fully active or nearly so after incubation of a crude cell extract at 70°C for 30 min. Surprisingly, perhaps, few of the other synthetases have any significant thermal stability in crude cell extracts. Further, the availability of *B. stearothermophilus* (Sargeant *et al.*, 1971) and of methods for the preliminary fractionation of its extracts (Atkinson *et al.*, 1972) makes feasible the isolation of thermostable synthetase on a reasonable scale. Since this work was begun the purification of several aminoacyl-tRNA synthetases from *B. stearothermophilus* has been reported. These include leucyl-tRNA synthetase (Vanhumbeeck & Lurquin, 1969; Lurquin, 1971),

isoleucyl-tRNA synthetase (Charlier & Grosjean, 1972) and arginyl-tRNA synthetase (Parfait & Grosjean, 1972). In all cases, as with the valyl-tRNA synthetase reported here, the enzymes have been found to be highly thermostable.

In the present work the properties of purified valyl-tRNA synthetases [L-valine-sRNA ligase (AMP); EC 6.1.1.9] from *E. coli* and from *B. stearothermophilus* have been compared. A method has been developed for the direct measurement of binding constants between aminoacyl-tRNA synthetases and tRNA, which should prove useful in studying the specific interactions between these two large molecules.

Experimental

Materials

B. stearothermophilus, strain NCA 1503, was supplied by the Microbiological Research Establishment (Porton Down, Salisbury, Wilts., U.K.), and stored frozen at -20°C until required. Valyl-tRNA synthetase from *E. coli* was prepared by J. Lapointe, J. R. Katze, D. Söll and J. R. Knowles by the method described for leucyl-tRNA synthetase (Hayashi *et al.*, 1970), and had a specific activity of 0.045 unit/mg of protein.

L-[¹⁴C]Valine (280 mCi/mmol) was supplied by The Radiochemical Centre (Amersham, Bucks., U.K.). tRNA from *E. coli* K12 CA265 was supplied in freeze-dried form by Whatman Biochemicals (Maidstone, Kent, U.K.). tRNA from *B. stearothermophilus* was prepared by the method of Zubay (1962) by Miss S. J. Putman. ATP (disodium salt:

grade 1) was supplied by Sigma (London) Chemical Co. Ltd. (Kingston, Surrey, U.K.)

DEAE-cellulose as DE23 and DE52, CM-cellulose as CM52, and phosphocellulose as P11, were supplied by W. and R. Balston Ltd. (Maidstone, Kent, U.K.). DEAE-Sephadex A-50 and A-25, and Sephadex G-200, were supplied by Pharmacia (G.B.) Ltd. (London, U.K.). Hydroxyapatite Bio-Gel HTP and Bio-Gel P150 (50–100 mesh) were supplied by Bio-Rad Laboratories Ltd. (St. Albans, Herts., U.K.). Hydroxyapatite was also prepared by the method of Main *et al.* (1959). All other chemicals were of the highest grade available.

Assay of enzymic activity

The normal method of assay was to measure the amount of ^{14}C -labelled amino acid incorporated into aminoacyl-tRNA in a given time of incubation. The assay solution (50 μl) contained: sodium cacodylate buffer, pH 7.2 (125 mM), magnesium acetate (12.5 mM), KCl (12.5 mM), ATP (2.5 mM), crude *E. coli* tRNA (0.5 mg/ml), L- ^{14}C valine (3 μM , 0.83 $\mu\text{Ci/ml}$), to which a suitable dilution of enzyme solution (10 μl) was added. [Where dilution of the enzyme solution was necessary, the buffer used was: sodium cacodylate buffer pH 7.2 (10 mM), glycerol (10%, v/v), bovine serum albumin (1 mg/ml), 2-mercaptoethanol (20 mM).] The assay mixture was incubated at 55°C for 10 min and then placed in an ice bath for 5 min. A portion (50 μl) was removed and placed on a Whatman 3MM filter paper (2 cm diam.), which was then immersed in an ice-cold solution of trichloroacetic acid in water (5%, w/v) and left for 15 min. Two repeat washings in trichloroacetic acid were followed by a wash in ethanol-ether (1:1, v/v) and then in ether. After drying in air, the filter-paper discs were subjected to scintillation counting. *E. coli* valyl-tRNA synthetase was assayed in the same way except that the incubation was at 37°C. When *B. stearothermophilus* crude tRNA was used a concentration that gave twice the normal $E_{260}^{10\text{mm}}$ was necessary to give the same initial rate of reaction. A final concentration having $E_{260}^{10\text{mm}} = 20$ was used in the assay. In all experiments using valyl-tRNA synthetases the enzyme was diluted to a concentration at which the assay was known to be linear, below 0.5 $\mu\text{g/ml}$ for the *B. stearothermophilus* enzyme, and about fivefold higher for the *E. coli* enzyme.

One enzyme-activity unit is defined as the amount that will incorporate 1 μmol of ^{14}C -labelled amino acid into aminoacyl-tRNA after 1 min of incubation under standard assay conditions.

Scintillation counting

The scintillation solvent contained: AnalaR toluene (500 ml), 2,5-diphenyloxazole (2 g), 1,4-bis-5-phenyloxazol-2-yl)benzene (50 mg). The counting

efficiency was 60% for ^{14}C -labelled amino acids under the conditions used.

Purification of the enzyme

All buffers used in the purification were made up with deionized water containing glycerol (10%, v/v) and 2-mercaptoethanol (20 mM). Purification steps were carried out at 4°C.

Crude extract. Cells (3 kg) were suspended in 100 mM-Tris-HCl buffer, pH 8.0 (3 litres), and crushed in an automatic French press at 0.2 GPa (30000 p.s.i.). The extract was then centrifuged for 60 min at 23000g, and the supernatant decanted. The pellet was re-extracted with the same buffer and re-centrifuged. The two supernatants were combined and applied to the first chromatography column with no prior dialysis or concentration.

DE23 chromatography. The column (9 litres) was equilibrated with 100 mM-Tris-HCl buffer, pH 7.0. The sample was applied and the column washed with about 25 litres of equilibration buffer until the $E_{280}^{10\text{mm}}$ of the solution emerging was less than 1.0. The valyl-tRNA synthetase was then eluted with the same buffer containing 300 mM-NaCl. Leucyl-tRNA synthetase was also eluted in this fraction. Arginyl-tRNA synthetase was not eluted until the buffer contained 1 M-NaCl. This buffer also eluted most of the nucleic acids, as judged by the E_{260} .

Hydroxyapatite chromatography. The column (2 litres, prepared as described by Main *et al.*, 1959) was equilibrated in 50 mM-potassium phosphate buffer, pH 6.8. The protein from the previous column was applied without dialysis, and eluted with a 20-litre linear gradient of the above buffer, increasing in concentration from 50 to 250 mM-potassium phosphate.

DE52 chromatography. The column (2 litres) was equilibrated in 10 mM-Tris-HCl buffer, pH 8.0, containing NH_4Cl (20 mM) and MgCl_2 (1 mM), and the fractions containing enzyme activity from the previous column were concentrated and dialysed into the same buffer. The sample was applied and the protein eluted with a 20-litre linear gradient of the above buffer containing additional NH_4Cl , 0–400 mM. Both valyl-tRNA synthetase and leucyl-tRNA synthetase were eluted near the middle of the gradient, and were pooled together.

CM52 chromatography. The column (1 litre) was equilibrated in 5 mM-sodium citrate buffer, pH 5.0. The sample was dialysed into the same strength of citrate solution at approx. pH 6.5, and the pH then lowered to 5.0 by the addition of 5 mM-citric acid immediately before application of the sample to the column. Protein was eluted with a 10-litre linear gradient of pH 5.0 citrate buffer, increasing from 5 to 50 mM. Leucyl-tRNA synthetase was completely separated from valyl-tRNA synthetase.

Hydroxyapatite chromatography. The column (200ml, commercial material) was equilibrated in 50mM-potassium phosphate buffer, pH6.8, and the valyl-tRNA synthetase from the previous column was dialysed into the same buffer. The column was subjected to a 2-litre linear gradient of buffer concentration increasing from 50 to 250mM.

DEAE-Sephadex chromatography. The column (200ml) was equilibrated in 50mM-potassium phosphate buffer, pH6.8, containing 50mM-KCl, and the enzyme was dialysed into the same buffer. Elution was by a 2-litre linear gradient of this buffer with KCl concentration increasing from 50 to 250mM.

Phosphocellulose chromatography. The column (500 ml) was equilibrated in 5mM-sodium citrate buffer, pH5.25. The sample was treated as for the CM52 column, and the pH adjusted to 5.25 immediately before application to the column. The protein was eluted with a 5-litre linear gradient of sodium citrate buffer increasing in concentration from 5 to 50mM, followed by a 5-litre linear gradient of 50mM-citrate buffer containing NaCl of concentration increasing from 0 to 450mM.

Hydroxyapatite chromatography. The column (450ml, commercial material) was equilibrated and the sample dialysed into 25mM-potassium phosphate buffer, pH6.8. Except for the lower starting concentration the 5-litre gradient was identical with that used for the previous hydroxyapatite column.

DE52 chromatography. The column (100ml) was equilibrated in 25mM-potassium phosphate buffer, pH6.5, and the sample dialysed into the same buffer. A linear gradient (1600ml) of this buffer containing from 0 to 300mM added KCl was applied.

The purified valyl-tRNA synthetase was stored at a concentration of about 1 mg/ml in triethanolamine-HCl buffer, pH7.5, containing glycerol (50%, v/v) and 2-mercaptoethanol (20mM), at 4°C.

Purification steps that were tested and found to give little increase in specific activity were a two-phase aqueous extraction in a mixture of dextran and polyethylene glycol in buffer (see Hayashi *et al.*, 1970), and a heat treatment.

Assay of protein concentration

Protein concentration was estimated by assuming that the $E_{280}^{1\text{cm}}$ of a 0.1% (w/v) solution of protein was 1. More accurate measurements of concentration were made by the method of Lowry *et al.* (1951). Samples were dialysed to remove 2-mercaptoethanol, made up to known volume, and the concentrations measured by use of at least five different dilutions.

Polyacrylamide-gel electrophoresis.

This was performed by the method of Davis (1964). The gels used for estimation of the homo-

geneity of protein preparations contained 7.5% (w/v) acrylamide and were run at pH8.5. Gels were stained with Naphthalene Black except when they were sliced and extracted for assay of enzymic activity.

Determination of molecular weight

This was done by using polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, as described by Shapiro *et al.* (1967). Protein samples were reduced and denatured in 2-mercaptoethanol and sodium dodecyl sulphate before analysis. Before staining, gels were soaked overnight in trichloroacetic acid (10%, w/v). The following standards were used: bovine serum albumin (mol. wt. 68000, 136000, 204000); catalase (mol. wt. 60000); ovalbumin (mol. wt. 43000); aldolase (mol. wt. 40000); yeast alcohol dehydrogenase (mol. wt. 37000); glyceraldehyde phosphate dehydrogenase (mol. wt. 36000); triose phosphate isomerase (mol. wt. 25000); and trypsin (mol. wt. 23300).

The method of Hedrick & Smith (1968) was also used to discover whether reduction and denaturation had split the protein into subunits. Gels of concentration 5, 6, 7, 8, 9 and 10% acrylamide were used.

pH-stability determination

The buffers used were 50mM, all containing glycerol (10%, v/v) and 2-mercaptoethanol (20mM). Those at pH4.75 and 5.8 were $\beta\beta$ -dimethylglutaric acid; at pH7.2 and 8.0, triethanolamine-HCl; at pH9.0 and 9.75, glycine; at pH11, NaHCO₃; and at pH12.6, KCl-KOH. Enzyme was added to each of these buffers. The *B. stearothermophilus* enzyme (10 μ g/ml) was incubated for 3 h at 55°C. The *E. coli* enzyme (4 μ g/ml) was incubated for 3 h at 37°C.

Variation in activity with change in pH of the assay

The buffers used in the assay were: $\beta\beta$ -dimethylglutarate at pH6.0, triethanolamine-HCl at pH7.0, 8.0 and 8.45, and glycine at pH9.2 and 10.0.

Measurement of binding constants of valyl-tRNA synthetase to tRNA

Crude *E. coli* tRNA was used in these experiments. Bio-Gel P150 was swollen in buffer and packed in a jacketed column (0.6cm $\times$ 65cm). The buffer used for column equilibration was: sodium cacodylate buffer, pH7.2 (125mM), magnesium acetate (12.5mM), KCl (12.5mM), crude tRNA (*E. coli*) (0.07mg/ml), 2-mercaptoethanol (20mM, added immediately before use). If the buffer was stored for any length of time, NaN₃ (10mM) was added.

Enzyme solution (20 μ l of a 3 mg/ml solution) was added to the above buffer (0.22 ml) and allowed to equilibrate. Approx. 150 ml of buffer was passed through the column to ensure complete equilibration. A few fractions were collected before the protein was applied, from which the background tRNA^{Val} concentration was measured. Exactly 0.2 ml of the enzyme solution was then applied to the column, leaving the remaining 40 μ l for accurate assays of enzymic activity. The enzyme was left on top of the column for about 5 min to ensure complete temperature equilibration, and then washed into the column with the equilibration buffer as carefully as possible to avoid unnecessary broadening of the enzyme peak. Fractions (1 ml) were collected. In all experiments the flow rate of the column was between 30 and 60 ml/h.

Enzymic activity was assayed by the normal method. Two portions (10 μ l) of the remaining enzyme pool were serially diluted to a concentration where accurate assays could be done (2500-fold dilution), and assays were performed in duplicate. Assays of the column fractions required only 120-fold dilution and were not duplicated as they were only used as a check on the pool assays, and as a measure of the overlap between the areas of increased and depleted tRNA concentration.

tRNA concentration was measured by using assay solution similar to that used for the enzyme assay, except that the tRNA was omitted, valyl-tRNA synthetase was included, and the solution was made more concentrated such that addition of 50 μ l of a column fraction to 100 μ l of assay mixture gave the normal concentrations of assay components. Incubation was for 20 min at 55°C. Control experiments had shown that this time was appropriate for maximum charging of the tRNA present. Two 50 μ l portions were taken from each assay for scintillation counting.

The column was freshly packed for each determina-

tion of binding constant at a different temperature to maintain the flow rate and to allow degassing of the column material.

Results and Discussion

Purification of valyl-tRNA synthetase from B. stearothermophilus

Valyl-tRNA synthetase was isolated from *B. stearothermophilus* by using the procedures shown in Table 1. Purification factors in Table 1 have been calculated from after the first purification step; thus a total purification of greater than 850-fold was achieved, with an overall yield of about 10% of the initial enzyme activity. The protein was 92% homogeneous as judged by densitometer scanning of a stained polyacrylamide gel, and had specific activity 1.28 units/mg.

Leucyl-tRNA and arginyl-tRNA synthetase activities were also followed during the purification. Arginyl-tRNA synthetase was separated from the other two on the first column, DE23 DEAE-cellulose at pH 7. Leucyl-tRNA synthetase and valyl-tRNA synthetase were separated on CM52 CM-cellulose at pH 5.0, and no leucyl-tRNA or arginyl-tRNA synthetase activity could be detected in the final valyl-tRNA synthetase preparation.

Isolation of valyl-tRNA synthetase from *B. stearothermophilus* on a smaller scale, but with a similar overall purification factor, has been reported by Charlier & Grosjean (1972). [Professor Grosjean has kindly informed us that the unit of enzyme activity in his paper should read nmol of charged tRNA formed in 10 min. In terms of the units used in the present work (μ mol of charged tRNA/min) the specific activity of the enzyme of Charlier & Grosjean (1972) is 1.99 units/mg, which compares well with our value of 1.28 units/mg (Table 1) determined under somewhat different conditions.]

Table 1. *Purification of valyl-tRNA synthetase from 3 kg of Bacillus stearothermophilus*

Full details are given in the Experimental section.

	Total protein (g)	Total E_{280}^{10mm}	Total activity (units)	Specific activity (units/mg of protein)	Increase in specific activity	Yield (%)
DEAE-cellulose (DE23), pH 7.0	76	110000	111	0.0015	1	100
Hydroxyapatite, pH 6.8	61	69000	121	0.002	1.3	100
DEAE-cellulose (DE52), pH 8.0	25	32000	90	0.0036	2.4	81
CM-cellulose (CM52), pH 5.0	2.8	2700	61	0.022	15	55
Hydroxyapatite, pH 6.8	0.5	1050	52	0.098	65	46
DEAE-Sephadex pH 6.8	0.2	190	35	0.151	100	33
Phosphocellulose (P11), pH 5.25	0.12	94.6	34	0.28	190	30
Hydroxyapatite pH 6.8	0.013	22.6	14	1.07	710	12
DEAE-cellulose (DE52), pH 6.5	0.009	20.2	12	1.28	850	11

Properties of valyl-tRNA synthetases from B. stearothermophilus and E. coli

The molecular weight of the valyl-tRNA synthetase from *B. stearothermophilus* was determined by using polyacrylamide-gel electrophoresis under reducing and denaturing conditions. The average of seven determinations gave the molecular weight as 110000 ± 4000 . That this value did not result from dissociation of subunits under the conditions used was shown by a separate molecular-weight determination by the method of Hedrick & Smith (1968). This demonstrated that the molecular weight under ordinary electrophoresis conditions lay between that of bovine serum albumin (68000) and that of aldolase (160000), and thus was not a multiple of 110000. The molecular weight of *E. coli* valyl-tRNA synthetase has also been reported to be 110000 with no evidence for subunit structure (Berthelot & Yaniv, 1970).

The properties of valyl-tRNA synthetase from *B. stearothermophilus* were compared with those of the *E. coli* enzyme. Both valyl-tRNA synthetases were tested to ensure that the assay conditions used were optimal. Mg^{2+} and ATP concentrations were varied, the ratio of the two being kept constant, and no change in initial rate was observed until the concentrations had been changed by about a factor of 2 in either direction, when in both cases activity fell. Increasing the tRNA concentration also had little effect on the rate. This applied to tRNA from both *E. coli* and *B. stearothermophilus*.

K_m (valine) was determined for both enzymes from the intercepts of plots of the reciprocal of the rate against the reciprocal of the valine concentration. The value for the *B. stearothermophilus* enzyme was $3.4 \times 10^{-5} M$, and that for the *E. coli* enzyme was $2.8 \times 10^{-5} M$.

Both valyl-tRNA synthetases lost no activity when incubated with 10mM-EDTA or with 6mM-*O*-phenanthroline. Leucyl-tRNA synthetase from *E. coli* is sensitive to EDTA (Hayashi *et al.*, 1970), but it appears to be the only aminoacyl-tRNA synthetase yet isolated which may be a metalloenzyme.

The pH-stability of both valyl-tRNA synthetases was determined and results are shown in Fig. 1. Both enzymes show their maximum stability at pH10, but above pH10 their activity decreases sharply and after 3 h at 37°C and pH11 the activity of the *E. coli* enzyme is almost completely destroyed. Under the same conditions the *B. stearothermophilus* enzyme remains stable, and only when the temperature is raised to 55°C does this enzyme lose activity at pH11. No such unusual stability to very alkaline pH values has been reported for other aminoacyl-tRNA synthetases, and it is possible that this property could be used to advantage in future purifications of the *B. stearothermophilus* enzyme. The activation of this enzyme after incubation at pH values between 8 and 10 is unexpected, but may arise from the

Fig. 1. pH-stability of valyl-tRNA synthetases

Valyl-tRNA synthetase from *B. stearothermophilus* (40 μ l of a 0.01 mg/ml solution) was added to 1.0 ml of buffer (for details see the Experimental section). One portion was assayed immediately and the remainder incubated for 3 h at 55°C and then assayed (●). The enzyme from *E. coli* (40 μ l of a 0.04 mg/ml solution) was incubated for 3 h at 37°C (○).

reduction by 2-mercaptoethanol of (an) essential thiol group(s). Most synthetases [with the exceptions of the lysyl-tRNA synthetase from *E. coli* (Stern & Peterkofsky, 1969) and the cysteinyl-tRNA synthetase from yeast (James & Bucovaz, 1969)], require the presence of thiol reagents for activity.

Both enzymes are very much less stable at low pH values, the *B. stearothermophilus* enzyme losing significant activity below pH7. In the purification of this enzyme, cation-exchange columns at pH values as low as pH5.0 were used without significant loss of activity, but in these cases the enzyme concentrations were much higher, and the columns were run at 4°C.

The pH-dependences of the assays for both valyl-tRNA synthetases are shown in Fig. 2. Both enzymes were maximally active between pH8.0 and 8.5. Up to pH8.5 the assay remains linear with time for up to 10 min; above this pH the rates decline rapidly with time, although initial rates remain high. Since the enzymes are stable up to pH10, this is very probably due to hydrolysis of the aminoacyl-tRNA produced (Hentzen *et al.*, 1972), which will be more significant for the *B. stearothermophilus* enzyme because of the higher temperatures used in its assay.

In all of the properties mentioned above there is a close similarity between the valyl-tRNA synthetases of *E. coli* and *B. stearothermophilus*; however, more differences between them might be expected in the temperature-dependence of their properties.

Not only are the temperature-stabilities of the two enzymes very different, but the rate at which activity loss increases with increasing temperature is very much slower for the *B. stearothermophilus* valyl-tRNA synthetase, as shown in Fig. 3. The *E. coli* enzyme shows a sharp fall in activity between 40 and 50°C under the conditions used, whereas the *B. stearothermophilus* enzyme, which starts to lose

Fig. 2. Variation in activity with the pH of assay

Valyl-tRNA synthetase from *B. stearothermophilus* (●) and from *E. coli* (○) was assayed at different pH values. For details see the text.

Fig. 3. Heat-stability of valyl-tRNA synthetase

Valyl-tRNA synthetase from *B. stearothermophilus* (2 µg/ml) was incubated at 55°C (●), 64°C (■) and 75°C (▲) for various times and then assayed normally at 55°C (a). The *E. coli* enzyme (8 µg/ml) was incubated at 20°C (○), 40°C (□), 50°C (△), 55°C (▽) and 60°C (●) for various times and then assayed normally at 37°C (b). For details see the text.

activity above 60°C, still loses activity fairly slowly at 75°C. Neither activity loss shows a simple kinetic pattern, so rate constants and activation energies for thermal denaturation cannot be calculated. Other published thermal-denaturation curves for synthetases (Charlier & Grosjean, 1972; Vanhumbeeck & Lurquin, 1969) follow the rapid inactivation pattern shown by the *E. coli* valyl-tRNA synthetase, which is typical of a co-operative process of high activation energy. In free-energy terms the difference between a mesophile and a thermophile protein in the binding energy maintaining the tertiary structure may not be very great. Let us assume that a mesophilic enzyme had a half-life under particular conditions of 1 week and its thermophilic counterpart had a half-life under the same conditions of 2 years, the activation free-energy difference would only be 11.7 kJ/mol (2.8 kcal/mol). In terms of the total stabilization energy of a protein this difference is trivial, and could amount, for instance, to a single extra hydrogen bond stabilizing the thermophile structure. These considerations point to there being some evolutionary advantage (possibly involving protein turnover, control, or the demands of catalysis) in having proteins of limited thermal stability and, perhaps, of greater conformational flexibility. It does seem that the half-lives of enzymes at the normal growth temperature of the organisms from which they are isolated are relatively similar, presumably because of an overriding metabolic need for a dynamic balance between enzyme synthesis and destruction. In the light of the free-energy differences involved, searches for characteristic differences in amino acid composition between mesophilic and thermophilic proteins (e.g. Sugimoto & Nosoh, 1971) may well be fruitless, since the differences in stability that are so important when handling proteins are very small energetically and (probably) structurally.

The temperature dependences of the activities of valyl-tRNA synthetases from *E. coli* and *B. stearothermophilus* were determined with tRNA from each source (Fig. 4). In these experiments, as in the study of the effect of tRNA concentration on the assay, no difference in the behaviour of the tRNA from the two sources was observed. This contrasts with the findings of Lurquin (1971) for the leucyl-tRNA from *E. coli* and *B. stearothermophilus*. *B. stearothermophilus* leucyl-tRNA synthetase activity decreased rapidly above 30°C with *E. coli* tRNA as substrate, but continued increasing up to 55°C with *B. stearothermophilus* tRNA. The conclusion was that small conformational changes in the *E. coli* tRNA caused the loss in activity between 30 and 50°C. However, no such difference is observed between the two valyl-tRNA species.

Arrhenius plots of log (initial rate) against the reciprocal of absolute temperature are non-linear for the *E. coli* enzyme (Fig. 4b), but almost linear up

Fig. 4. Effect of temperature on the activity of valyl-tRNA synthetase

(a) The effect of temperature on the activity of *B. stearothermophilus* valyl-tRNA synthetase with *B. stearothermophilus* tRNA (●), and with *E. coli* tRNA (○). (b) Effect of temperature on the activity of *E. coli* valyl-tRNA synthetase with *B. stearothermophilus* tRNA (●), and with *E. coli* tRNA (○).

to 50°C for the *B. stearothermophilus* enzyme, corresponding to an apparent activation energy of 42 kJ/mol (10 kcal/mol) (Fig. 4a).

Measurement of binding constants of tRNA to aminoacyl-tRNA synthetase

Preliminary experiments were carried out to develop a direct method of studying the binding of tRNA synthetase to its cognate tRNA. The method of Fairclough & Fruton (1966) was adapted, a Bio-Gel P150 column being used to separate the synthetase and synthetase-tRNA complex from free tRNA. The column was equilibrated in a solution containing tRNA, and a solution containing enzyme in the same concentration of tRNA was passed down the column. Assays for both enzyme and tRNA showed a peak of tRNA concentration corresponding to the enzyme peak, followed by a dip in the tRNA concentration

Fig. 5. Elution of *B. stearothermophilus* valyl-tRNA synthetase from a Bio-Gel P150 column equilibrated with tRNA

The column (0.6×65 cm) was run at 18°C. Assay of enzyme (----); assay of tRNA (—); correction to tRNA peak to allow for overlap with the region of depleted tRNA (····). For details see the text.

Table 2. Binding constants for crude *E. coli* tRNA to *B. stearothermophilus* valyl-tRNA synthetase

For details see the text.

Binding constant (M ⁻¹)	Temperature (°C)
8.6 × 10 ⁶	5
0.90 × 10 ⁶	18
0.45 × 10 ⁶	40
0.35 × 10 ⁶	60

where the solution depleted in tRNA by its binding to the synthetase emerged from the column. If the peak and dip are completely separated then their areas can be used, in conjunction with the known concentration of enzyme and of tRNA in the original solution, to calculate the binding constant of tRNA to enzyme.

Results of a typical run are shown in Fig. 5. Complete separation of enzyme from tRNA was not always achieved, but the area of the peak could be corrected for overlap with the depleted area, since the ratio of enzyme to bound tRNA should be constant over the peak. This ratio was calculated for the first two peak fractions and then used to correct that of the third.

Results of four such runs at temperatures between 5 and 60°C using *B. stearothermophilus* valyl-tRNA synthetase and crude *E. coli* tRNA are shown in Table 2. Fig. 6 is a plot of log (binding constant) against the reciprocal of absolute temperature, and shows the sharp fall in binding constant between 5 and 18°C. Between 18 and 60°C the binding constant decreases slowly and the graph is almost linear. The

Fig. 6. Plot of $\log$ (binding constant of tRNA) against the reciprocal of the absolute temperature for valyl-tRNA synthetase from *B. stearothermophilus*

For details see the text.

sharp fall may be due to a conformational change in either enzyme or tRNA between 5 and 18°C.

Other methods used to measure binding constants between synthetases and tRNA include the adsorption of synthetase but not of tRNA on nitrocellulose filters (Yarus & Berg, 1967), quenching of protein fluorescence caused by tRNA binding (Rigler *et al.*, 1970) and protection of the enzyme by tRNA against heat inactivation (Charlier & Grosjean, 1972). The present method is less likely to perturb the binding constant than is the nitrocellulose-filter method, and is more universally applicable and direct than the other methods. It is also easily adapted to measurement at different temperatures.

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