Affinity Chromatography of Aminoacyl-Transfer Ribonucleic Acid Synthetases

COGNATE TRANSFER RIBONUCLEIC ACID AS A LIGAND

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The use of tRNA affinity columns for the purification of aminoacyl-tRNA synthetases was investigated. A purification method for valyl-tRNA synthetase from *Bacillus stearothermophilus* is described that uses two affinity columns, one containing the pure cognate tRNA, and the other containing all tRNA species except the cognate tRNA. A method for the rapid preparation of the two columns was developed, which does not require prior isolation of cognate tRNA but makes use of the ability of the target synthetase to select its cognate tRNA. The usefulness of tRNA columns is compared with that of affinity columns derived from the aminoalkyladenylate reported in the preceding paper [Clarke & Knowles (1977) *Biochem. J.* 167, 405-417].

The successful purification of an aminoacyl-tRNA synthetase on an affinity column of the pure cognate tRNA would be predicted on the basis of the strength of binding involved [K_{diss} for tRNA-synthetase is commonly about 10 nM (Söll & Schimmel, 1974)], and the high specificity with which a synthetase recognizes its cognate tRNA. However, several reports of synthetase purification with such columns have been disappointing (Nelidova & Kisselev, 1968; Bartkowiak & Pawelkiewicz, 1972; Hayashi, 1973), and the purification factors that were obtained (about 30-fold) hardly justify the labour of isolating the purified tRNA required as the affinity-column ligand. Remy *et al.* (1972) achieved a similar result in their purification of yeast phenylalanyl-tRNA synthetase on a column of tRNA^{Phe}. The enzyme contained some contaminants that had also bound to the tRNA^{Phe} and were probably enzymes involved in other aspects of RNA metabolism. In contrast with the aminoacyl-tRNA synthetase, which recognizes specific features of a single tRNA (or group of isoaccepting tRNA species), such enzymes would recognize general features of all tRNA species. Remy *et al.* (1972) therefore proposed a method involving two affinity columns: one of pure cognate tRNA, preceded by one of tRNA lacking the cognate tRNA, this latter to remove other tRNA-binding proteins. In this way, yeast phenylalanyl-tRNA synthetase was purified to homogeneity and rat liver seryl-tRNA synthetase to 85-90% purity (Befort *et al.*, 1974).

The preparation of the necessary pair of affinity columns requires the separation of cognate from

non-cognate tRNA. This has hitherto been achieved by classical methods. Yet a particular aminoacyl-tRNA synthetase makes this very distinction (between cognate and non-cognate species) in its interaction with tRNA, and it is possible to exploit this fact to effect the desired separation, thus avoiding a laborious fractionation of tRNA by physical techniques. A further saving in time and effort is achieved by using the enzymic aminoacylation reaction to discriminate between cognate and non-cognate tRNA during the sequence of reactions in which tRNA is coupled to the column material. Moreover, this method does not require a pure enzyme preparation, since complete specificity for the cognate tRNA can be achieved simply by adding a single amino acid to a mixture of crude synthetases and crude tRNA.

The reaction sequence for the preparation of columns designed for the purification of valyl-tRNA synthetase is detailed in Scheme 1. The use of the enzymic aminoacylation reaction in conjunction with periodate oxidation to differentiate between cognate and non-cognate tRNA is not without precedent: several methods for the purification of tRNA are based on this principle (e.g., Zubay, 1962; Zamecnik *et al.*, 1960).

Successful application of this approach to the purification of a particular synthetase requires as little overlap in specificity as possible between the two affinity columns, so that one must minimize the extent to which the non-cognate tRNA column contains cognate tRNA and vice versa. Cognate tRNA may be incorporated into the non-cognate tRNA column as a result of incomplete aminoacylation, loss of the aminoacyl group from aminoacyl-tRNA before or during oxidation, or coupling of

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non-oxidized tRNA to the column material. Non-cognate tRNA may appear in the cognate tRNA column if the oxidation of non-cognate tRNA is not quantitative, or if a portion of the oxidized tRNA fails to bind to the Sepharose. Further problems may arise as a result of misrecognition and/or mischarging by a synthetase of an incorrect tRNA.

In the present paper we report experiments carried out to establish the feasibility of the proposed method of preparation of the two affinity columns, and the use of such columns in the purification of valyl-tRNA synthetase from *Bacillus stearothermophilus*. Finally, the usefulness of tRNA columns is assessed in relation to the small-molecule systems discussed in the preceding paper (Clarke & Knowles, 1977). A preliminary report of some of this work has already appeared (Joyce & Knowles, 1974).

Experimental

Materials

tRNA from *Escherichia coli* K12 was purchased as a freeze-dried solid from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Inorganic pyrophosphatase (EC 3.6.1.1) (type III) from baker's yeast (freeze-dried powder) and adenylate kinase (EC 2.7.4.3) (grade III) from rabbit muscle [(NH₄)₂SO₄ suspension] were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. A partially purified mixture containing valyl-, leucyl- and tyrosyl-tRNA synthetases from *B. stearothermophilus* was kindly given by Dr. K. Sargeant and Dr. A. Atkinson, Microbiological Research Establishment, Porton Down, Salisbury, Wilts., U.K.

Enzyme assay

B. stearothermophilus aminoacyl-tRNA synthetases were assayed as described by Wilkinson & Knowles (1974) except that triethanolamine/HCl buffer, pH 7.2, was used instead of cacodylate at the same pH. A unit of enzyme activity is defined as the amount that will incorporate 1 μ mol of ¹⁴C-labelled amino acid into aminoacyl-tRNA after 1 min incubation under standard assay conditions.

tRNA assay

tRNA was assayed by measuring the incorporation of the relevant ¹⁴C-labelled amino acid into aminoacyl-tRNA, after sufficient time to allow the aminoacylation reaction to reach a steady state. The assay mixture was the same as for the enzyme assay except that tRNA was omitted, and sufficient crude enzyme solution included to provide approx. 100 μ units of the relevant synthetase/ml. To a portion (50 μ l) of this solution was added the unknown tRNA solution (10 μ l). After 30 min incubation at 55°C, the filter-disc procedure of the enzyme assay was followed. Since the degree of aminoacylation of tRNA is

strongly dependent on the exact reaction conditions (as discussed below), assays of an unknown tRNA solution were always accompanied by assays of a series of standard solutions of crude *E. coli* tRNA. Interpolation on the calibration curve so obtained allowed calculation of the extent to which the concentration of a particular tRNA species had been increased or decreased relative to the situation in unfractionated tRNA.

Time course for tRNA aminoacylation

The reaction mixture contained the same components as the enzyme assay mixture, although the concentrations were varied, and in some cases additional coupling enzymes were present, to give optimum aminoacylation. Samples (50 μ l) were withdrawn at timed intervals during incubation at the chosen temperature. These were placed on filter-paper discs which were processed as for the enzyme assay.

Preparation of valyl-tRNA

Valyl-tRNA was prepared as described[†] by Joyce & Knowles (1974). The precipitated tRNA was dissolved in cold 0.1 M-sodium acetate buffer, pH 5, containing 10 mM-MgCl₂, and dialysed against this buffer.

[¹⁴C]Valyl-tRNA was prepared in an identical manner with a lower concentration (2.5 μ M) of [¹⁴C]-valine (265 mCi/mmol).

Stability of valyl-tRNA

[¹⁴C]Valyl-tRNA was prepared as described above. The loss in radioactivity from the tRNA was measured by removal at timed intervals of samples (50 μ l) from the incubation medium. These were placed on filter-paper discs, which were processed as for the enzyme assay.

Oxidation of tRNA

Periodate oxidation of tRNA was carried out as described by Remy *et al.* (1972). The resulting tRNA_{ox}* solution was dialysed exhaustively at 4°C against 0.1 M-sodium acetate, pH 5, containing 10 mM-MgCl₂.

Preparation of tRNA_{ox}-red.

NaBH₄ reduction of tRNA_{ox} was based on the method of RajBhandary (1968), as described by Joyce & Knowles (1974).

Indirect measurement of the extent of aminoacylation of tRNA

The extent of aminoacylation of tRNA^{val} was

* Abbreviations: tRNA^{-val}, all tRNA species other than valine isoacceptors; tRNA_{ox}, tRNA oxidized at the 3'-terminal adenosine to the corresponding dialdehyde; tRNA_{ox}-red., tRNA_{ox} reduced by borohydride to the bis(primary alcohol) at the 3'-terminal adenosine.

determined by measuring the extent to which tRNA^{Val} in total tRNA was protected against periodate oxidation. This method was particularly useful when direct measurement by tracer methods was inappropriate, for example when investigating the effect of high concentrations of amino acid on aminoacylation.

Crude *E. coli* tRNA (2mg) was charged with valine as described by Joyce & Knowles (1974) (except that various amino acid concentrations were used when determining the effect on charging). After oxidation, the valyl-tRNA was deacylated by dialysis against 0.1M-glycine/NaOH buffer, pH10.3, at room temperature (20°C) for 5h. It was then dialysed into 10mM-triethanolamine/HCl buffer, pH7.2, containing MgCl₂ (10mM) before assay for tRNA^{Val} activity. Comparison of the assay results with those obtained for a series of crude *E. coli* tRNA solutions of known concentration gave the percentage of the original tRNA^{Val} activity remaining in the sample. (A control sample, treated similarly except that periodate was omitted from the oxidation medium, retained all its tRNA^{Val} activity, showing that no activity loss was associated with the experimental procedures, apart from the periodate oxidation itself.)

Preparation of tRNA columns

Hydrazinyl-Sepharose. Commercial CNBr-activated Sepharose 4B (Pharmacia Ltd., Uppsala, Sweden) was prepared for use as described in the manufacturer's instructions and derivatives were prepared with hydrazine as described by Cuatrecasas (1970).

Sepharose-adipic acid dihydrazide. Adipic acid dihydrazide was prepared and coupled to CNBr-activated Sepharose 4B as described by Lamed *et al.* (1973).

Attachment of unfractionated tRNA to Sepharose. Crude *E. coli* tRNA was oxidized as described above. *A*₂₆₀ measurements on the final dialysis residue showed about 80% recovery of tRNA from the oxidation procedure. To this material (approx. 10ml of a 4mg/ml solution of tRNA) in 0.1M-sodium acetate buffer, pH5, was added Sepharose-adipic acid dihydrazide previously equilibrated with the same buffer. The mixture was incubated for 1h at 37°C, and then shaken overnight at room temperature. The gel was recovered by filtration and washed with 1M-NaCl until the washings showed negligible absorbance at 260nm.

The difference in u.v. absorption between the original oxidized tRNA solution and the recovered washings gave the extent of coupling, typically 3–5mg of tRNA/ml. Alternatively, the amount of tRNA (as *A*₂₆₀) released from the gel after prolonged alkaline hydrolysis was measured by the method of Hayashi (1973). When a single batch of column

material was assessed by both methods, the results were in close agreement.

Preparation of tRNA^{Val} and tRNA^{-Val} columns. Crude *E. coli* tRNA (40mg) was charged with valine as described by Joyce & Knowles (1974). A small quantity of a similar reaction mixture containing, in addition, [¹⁴C]valine (15μM, 4.5μCi/ml), was incubated at the same time, and samples (50μl) were withdrawn at 30min intervals to check that the reaction was proceeding satisfactorily.

The charged tRNA was oxidized and coupled to Sepharose-adipic acid dihydrazide (6ml) as described above for unfractionated tRNA. Typically 70–80% of the tRNA was coupled, giving about 4mg of tRNA^{-Val}/ml of Sepharose.

After removal of the gel by filtration, and washing with 1M-NaCl, the tRNA-containing washings were dialysed exhaustively against water, and the tRNA was recovered by freeze-drying. This material (approx. 5mg) was treated with NaBH₄ as described previously (Joyce & Knowles, 1974) to reduce any residual tRNA_{ox}. The valyl-tRNA^{Val} was deacylated by dialysis against 0.1M-glycine/NaOH buffer, pH10.3, at room temperature for 5h.

After dialysis into 10mM-triethanolamine/HCl, pH7.2, containing MgCl₂ (10mM), the tRNA was assayed for tRNA^{Val} (to determine the extent to which this tRNA had been purified from non-cognate tRNA species) and for tRNA^{Leu} (to confirm that no active non-cognate tRNA remained at this stage). The tRNA was then dialysed into 0.1M-sodium acetate, pH5, before oxidation and coupling of tRNA^{Val} to Sepharose-adipic acid dihydrazide (1ml) in the usual manner, yielding a column material carrying 1mg of tRNA^{Val}/ml.

Investigation of binding capacities of tRNA column materials for synthetases

The column material was equilibrated with 0.05M-sodium acetate buffer, pH5.5, containing glycerol (10%, v/v), MgCl₂ (10mM), EDTA (1mM) and 2-mercaptoethanol (20mM). A sample (approx. 0.1ml) was allowed to equilibrate at 0°C with an impure mixture of synthetases in the same buffer. Non-bound material was removed by filtration, and assayed to ensure that the column material was saturated with the relevant synthetases. The gel was washed with the original buffer (6×1ml), and then bound synthetases were eluted with either 0.05M-sodium acetate, pH5.5 (as above) containing 1M-KCl, or 0.1M-Tris/HCl, pH8, containing 1M-KCl. The washings (3×1ml) were assayed for various synthetases (usually valyl-, leucyl- and tyrosyl-tRNA synthetases). Summation of the assay results for the three fractions gave the total quantity of each enzyme bound to the column material, expressed as radioactivity (c.p.m.) incorporated into tRNA under standard assay conditions with a portion (10μl) of

the washings. The comparative treatment of the results (Table 2) made it unnecessary either to relate the assay results to actual quantities of protein or to use an accurately determined quantity of gel.

Elution of tRNA columns

The columns were eluted with 0.05M-sodium acetate, pH 5.5, containing glycerol (10%, v/v), $MgCl_2$ (10mM), EDTA (1mM) and 2-mercapto-ethanol (20mM). After application of the sample and washing of the column with starting buffer to remove non-bound protein, bound protein was eluted with a linear gradient of KCl (0–1M) in the same buffer. Because of the instability of aminoacyl-tRNA synthetases at low pH, fractions were collected into tubes containing sufficient 1M-triethanolamine/HCl, pH 7.2, to raise the pH to about 7. Pooled fractions were concentrated by vacuum dialysis.

Gel electrophoresis

Disc polyacrylamide-gel electrophoresis was carried out by the method of Davis (1964), with polyacrylamide gels run at pH 8.5 and at 3 mA/tube. Gels were stained with Coomassie Blue.

Results and Discussion

Aminoacylation of tRNA

The success of the reaction scheme proposed for the preparation of tRNA columns (Scheme 1) depends on the aminoacylation of tRNA proceeding as nearly as possible to completion. However, the progress curve for tRNA aminoacylation (measured by the incorporation of ^{14}C -labelled amino acid into tRNA) reaches a plateau value that corresponds neither to complete aminoacylation nor to a true enzyme-catalysed equilibrium, since the plateau value is dependent on enzyme concentration (Fig. 1). Bonnet & Ebel (1972) have investigated the causes of incomplete aminoacylation in the system yeast tRNA^{Val}/valyl-tRNA synthetase. They concluded that, under normal reaction conditions, the aminoacylation plateau reflects a balance between four competing processes (Scheme 2): the expected enzyme-catalysed forward and reverse reactions (*a* and *b*), a non-enzymic hydrolysis of aminoacyl-tRNA (*c*), and an enzyme-catalysed hydrolysis of aminoacyl-tRNA (*d*). Scheme 2 accounts for the dependence of the plateau value on enzyme concentration, since a change in enzyme concentration will alter the rates of the enzyme-catalysed processes without affecting the non-enzymic hydrolysis. Aminoacylation is also sensitive to changes in the reaction medium, in particular the ionic strength, and the conditions were adjusted (as described below) so as to increase the rate of the forward reaction and/or decrease the importance of one or more of the reverse reactions.

Fig. 1. Effect of enzyme concentration on aminoacylation

The incubation mixture contained: triethanolamine/HCl buffer (104mM), pH 7.2; magnesium acetate (10.4mM); KCl (10.4mM); ATP (2.1mM); valine (2.5μM); tRNA (0.42mg/ml). Valyl-tRNA synthetase concentrations: ○, 10μunits/ml; ●, 50μunits/ml; □, 250μunits/ml. Incubation was at 55°C.

Enzyme and tRNA concentration. Bonnet & Ebel (1972) have shown that an improvement in aminoacylation should result if the enzyme concentration is increased. However, raising aminoacylation by increasing the enzyme concentration is not very useful when the eventual purpose of the experiment is an enzyme purification, and an enzyme concentration of 50 μunits/ml was chosen as an upper limit, thus giving reasonable aminoacylation (Fig. 1), without being unnecessarily wasteful.

To gain the maximum benefit from this enzyme concentration it was necessary to have as high a concentration of tRNA as possible, consistent with the specific aminoacylation remaining high. It was assumed that, as long as the plateau value varied linearly with tRNA concentration (so that the same percentage of tRNA was being charged), this value could be further improved by altering experimental conditions. The tRNA concentration can be increased to 1.67mg/ml and still continues to satisfy this criterion (Fig. 2). Higher concentrations were not tested.

pH and temperature. Aminoacylation was insensitive to changes in pH between 7 and 8. Clearly the faster rate of the enzyme-catalysed reaction at the higher pH is counterbalanced by the increase in the spontaneous hydrolysis of aminoacyl-tRNA. The use of pH values outside this range is inadvisable, owing to the slower enzyme-catalysed reaction at acidic pH and the more rapid hydrolysis of aminoacyl-tRNA in alkaline solutions.

Initially the reaction was carried out at 55°C, the optimum temperature for the *B. stearothermophilus* enzyme. Lowering the temperature to 40°C increased aminoacylation, presumably because the non-enzymic hydrolysis was slowed more than the enzyme-catalysed process. Further lowering of the temperature to 30°C did not effect any additional improvement.

Substrate concentrations. The plateau value was

Scheme 1. Preparation of affinity columns

Sepharose~~~~tRNA^{Val} contains tRNA^{Val} linked to Sepharose 4B. Sepharose~~~~tRNA^{-Val} contains all tRNA species except tRNA^{Val}, linked to Sepharose 4B.

Scheme 2. Competing reactions in the aminoacylation of tRNA

Fig. 2. Effect of tRNA concentration on aminoacylation

The conditions were as for Fig. 1, with valyl-tRNA synthetase at 50 μ units/ml and tRNA concentrations of 0.42 (○), 0.84 (●) and 1.67 (□) mg/ml.

insensitive to variations in the Mg^{2+} concentration (between 10 and 135 mM) and to doubling the ATP concentration. However, the extent of the aminoacylation reaction was particularly sensitive to deterioration of the ATP solution, increased charging being observed when fresh solutions were used. Since the reaction rate is unaffected by the concentration of ATP, this cannot simply be the result of decreased ATP concentration caused by decomposition, and a more likely explanation is that a decomposition product of ATP (possibly AMP from hydrolysis and/or disproportionation) adversely affects aminoacylation.

The amino acid concentration was increased from 2.5 μ M (the concentration used in the enzyme assay) to cope with the larger amount of tRNA in the reaction mixture. In the presence of 0.83 mM-valine, 80–90% aminoacylation was obtained (as measured by the percentage protection of tRNA^{Val} against oxidation by periodate).

Use of coupling enzymes. No further improvement in charging was achieved by the use of coupling

enzymes, pyrophosphatase and adenylate kinase, to remove the products pyrophosphate and AMP respectively, which may act as substrates in the reverse reaction.

Stability of aminoacyl-tRNA. Studies with [^{14}C]-valyl-tRNA demonstrated the stability of the aminoacyl-tRNA bond under various conditions. No appreciable loss of radioactivity from tRNA occurred during 5 h at room temperature in the periodate-oxidation medium; normally only 1 h reaction time is required. Aminoacyl-tRNA species are most stable to hydrolysis under conditions of low pH and low temperature (Hentzen *et al.*, 1972), and care was taken to ensure that such conditions prevailed at all times during the isolation of charged tRNA before oxidation. ^{14}C -labelled aminoacyl-tRNA was also used to check the efficiency of the deacylation reaction.

Oxidation of tRNA and coupling to Sepharose derivatives

The periodate-oxidation reaction was investigated by oxidation of mixed tRNA and subsequent isolation and assay for tRNA^{Val} . The oxidized tRNA had less than 5% of the expected acceptor activity, and a control sample (subject to the same experimental conditions, but omitting the periodate) retained all its tRNA^{Val} acceptor activity. On the basis that other tRNA species were similarly affected by periodate, this experiment showed that the oxidation reaction proceeded more than 95% to completion.

Coupling of oxidized tRNA to hydrazine groups on the carrier matrix was investigated by using oxidized mixed tRNA; the extent of coupling was calculated from the decrease in A_{260} of the aqueous phase. Coupling yields of about 90% were typical (Table 1).

Coupling of non-oxidized tRNA to the carrier

Table 1. *Coupling of oxidized tRNA to derivatized Sepharose*
For details, see the text.

Coupled to	tRNA added (mg/ml of gel)	tRNA coupled (mg/ml of gel)	Coupling yield (%)
Sepharose–adipic acid dihydrazide	11.6	10.4	90
	8.4	7.4	88
	4.6	4.4	95
	2.3	2.1	91
Hydrazinyl-Sepharose	11.6	11.0	95
	8.4	7.8	93
	4.6	4.4	95
	2.3	2.2	95

matrix should not present a serious problem, since it has been shown that, under the conditions used, less than 5% of non-oxidized tRNA is bound to the matrix (Remy *et al.*, 1972).

As Scheme 1 shows, the small amount of oxidized non-cognate tRNA that escapes attachment to the column material would be capable of participating in subsequent reactions, eventually becoming coupled to the cognate tRNA column. This can be prevented if, after removal of the Sepharose containing the non-cognate tRNA, the recovered tRNA is treated with NaBH_4 to convert the dialdehyde (tRNA_{ox}) into the diol ($\text{tRNA}_{\text{ox-red}}$) (Cramer *et al.*, 1968; RajBhandary, 1968), which is inactive in subsequent steps. A control experiment showed that NaBH_4 has no adverse effect on non-oxidized tRNA.

Preparation and preliminary assessment of tRNA columns

Columns containing bound tRNA^{Val} and bound $\text{tRNA}^{-\text{Val}}$ designed for the purification of valyl-tRNA synthetase were prepared as outlined in Scheme 1, incorporating the procedures chosen on the basis of the experiments discussed above.

Before oxidation and coupling to produce the tRNA^{Val} column, the tRNA solution was assayed both for tRNA^{Leu} (to confirm that non-cognate tRNA has been removed or inactivated during the previous stages) and for tRNA^{Val} . Typically, the amount of tRNA^{Val} present (as a percentage of total tRNA) was between 2- and 3-fold greater than in the original solution of mixed tRNA. If the tRNA^{Val} were pure at this stage, this value would be in excess of 10-fold, and the fact that it was lower indicates the presence of non-cognate tRNA that escaped coupling to the first affinity column. Since the latter will be present at this stage as $\text{tRNA}_{\text{ox-red}}$, that cannot couple to the column material, these assay

results do not preclude the production of a substantially pure tRNA^{Val} column.

A rapid estimate of the relative binding capacities of these column materials for different synthetases was made by equilibration of a small quantity of the column material with excess of a mixture of synthetases under conditions favourable to strong binding between enzyme and cognate tRNA (pH 5.5, in buffer of low ionic strength). After the Sepharose had been washed, the bound enzyme was eluted by raising either the pH or the ionic strength. (Under the same conditions the binding of synthetases to unsubstituted Sepharose–adipic acid dihydrazide was negligible, demonstrating the absence, in this system, of non-specific interactions with the spacer group.) The efficiency of the tRNA^{Val} and $\text{tRNA}^{-\text{Val}}$ columns was assessed by comparing the relative amounts of the various synthetases bound to these column materials, with corresponding values obtained for total tRNA.

The results (Table 2) show that contamination of the tRNA^{Val} column with non-cognate tRNA species occurs only to a minute extent. The values for the $\text{tRNA}^{-\text{Val}}$ column, which suggest that this column contains substantial amounts of tRNA^{Val} , seem incompatible with the high charging values (80–90%) achieved in the aminoacylation reaction. However, it seems likely that this discrepancy is the result of misrecognition, as discussed below.

Remy *et al.* (1972) found it necessary to stabilize the bond between tRNA_{ox} and their hydrazinyl column matrix. However, the bond between tRNA_{ox} and an immobilized acyl hydrazide is sufficiently stable to make this unnecessary (Lamed *et al.*, 1973; Hansske *et al.*, 1974). In our hands, the omission of this step made no difference to the behaviour of our column materials, as judged by stability under storage (pH 5, 4°C), and by the binding capacity for synthetases.

Purification of valyl-tRNA synthetase

Choice of elution conditions. It is well known that the synthetase–tRNA interaction is strongest at low pH (Yarus & Berg, 1969; Pachmann *et al.*, 1973; Lam & Schimmel, 1975), and is weakened by increases in ionic strength. After loading our tRNA columns with enzyme at pH 5.5 and washing with low-ionic-strength buffer to remove unbound protein, elution of bound synthetases could be accomplished by use of a salt gradient (0–1 M-KCl) at this pH (as described in the Experimental section). Pachmann *et al.* (1973), in studying the enzyme–tRNA interaction in free solution, concluded that the specificity of the interaction is increased as the binding is weakened, for example by raising the pH. Similarly, Hayashi (1973) found that elution of a tRNA^{Leu} column at pH 7 gave optimum purification of leucyl-tRNA synthetase. However, elution of our columns

Table 2. *Binding of synthetases to tRNA columns*

Total enzyme bound was measured as radioactivity (c.p.m.) incorporated into tRNA under standard assay conditions, normalized to 100 for valyl-tRNA synthetase.

Column ligand	Total enzyme bound		
	Valyl-tRNA synthetase	Leucyl-tRNA synthetase	Tyrosyl-tRNA synthetase
Total tRNA	100	184	187
tRNA ^{-Val}	100	236	
tRNA ^{Val}	100	8	9
tRNA ^{-Leu}	100	26	
tRNA ^{Leu}	100	194	12

with buffers (50mM-sodium phosphate) at pH6.25 and 7 sharply decreased binding of all synthetases. Presumably, the 3'-terminus of tRNA is important in the synthetase-tRNA interaction, contributing to binding strength without contributing to specificity, since all tRNA species have the same trinucleotide sequence at this point. This structural feature is not available for interaction with the enzyme when tRNA columns are prepared by periodate oxidation at the 3'-terminus.

On the basis of these preliminary experiments, a salt gradient at pH5.5 (as described in the Experimental section) was chosen as the most satisfactory elution procedure. Chromatography of an impure enzyme solution on a tRNA^{Val} column under these conditions (see, e.g., Fig. 1 of Joyce & Knowles, 1974) showed a much higher capacity for valyl-tRNA synthetase compared with other synthetases. (Thus the ratio of valyl-tRNA synthetase/tyrosyl-tRNA synthetase eluted by the salt gradient was 14:1 compared with a ratio of 0.77:1 on a column of mixed tRNA.) Gel electrophoresis of the pooled fractions from the tRNA^{Val} column showed three major components (Fig. 3b). This result was entirely expected since, as mentioned above, a single column of cognate tRNA is incapable of separating the target enzyme from other tRNA-binding proteins.

Improved purification was achieved by first subjecting the crude mixture to chromatography on a column (0.7cm×9cm) of tRNA^{-Val} under the conditions described in the Experimental Section: the enzyme sample was applied and the column washed with starting buffer to remove unbound protein. This material was pooled and concentrated about 6-fold by vacuum dialysis; assays showed the expected increase in valyl-tRNA synthetase activity relative to other synthetases. A suitable quantity of this pooled material was then chromatographed on the tRNA^{Val} column (0.7cm×2.5cm) (see Fig. 2 of Joyce & Knowles, 1974); polyacrylamide-gel electrophoresis of the fractions containing valyl-tRNA synthetase activity now showed one major component (Fig. 3c). Extraction and assay of slices of a gel of the original

crude mixture confirmed that valyl-tRNA synthetase migrates to this position.

Problems arising from misrecognition. tRNA^{Leu} and tRNA^{-Leu} columns, analogous to the columns described above, were prepared and their capacities for various synthetases were determined (Table 2). A surprisingly large amount of valyl-tRNA synthetase is bound to the tRNA^{Leu} column, and this is probably due to misrecognition of tRNA^{Leu} by this synthetase. The same misrecognition accounts for the much less effective exclusion of valyl-tRNA synthetase from the tRNA^{-Val} column (Table 2), compared with the exclusion of leucyl-tRNA synthetase from the tRNA^{-Leu} column. Moreover, the tRNA^{Leu} solution before oxidation and coupling to the second column showed valine-acceptor activity in the tRNA^{Val} assay. At this stage of the preparation, the only tRNA^{Val} species present should be tRNA^{Val}_{ox.-red.}. Although aminoacylation of such a species has been observed in one instance (von der Haar *et al.*, 1971), the absence of amino acid acceptance in our tRNA^{Val}_{ox.-red.} was demonstrated with mixed tRNA_{ox.-red.} in the tRNA^{Val} assay system. This observation may also be explained by misrecognition and mischarging of a tRNA^{Leu} by valyl-tRNA synthetase. This hypothesis does not conflict with the lack of similar mischarging in the initial stages of the tRNA^{Val}-column preparation, since it has been shown that a particular mischarging reaction may take place with a substantially pure tRNA and yet be suppressed by competition from the cognate tRNA when unfractionated tRNA is the substrate (Giegé *et al.*, 1974).

Similar problems due to enzyme-tRNA misrecognition were encountered by Befort *et al.* (1974) during the purification of rat liver seryl-tRNA synthetase on tRNA^{Ser} and tRNA^{-Ser} columns. The target enzyme bound strongly to the tRNA^{-Ser} column, owing to misrecognition and binding to one or more non-cognate tRNA species. The problem was much more severe when yeast tRNA was used as the source of tRNA^{-Ser}, rather than rabbit liver tRNA. This illustrates the general observation that such errors in

Fig. 3. Densitometer scans from polyacrylamide gels of synthetase samples

(a) Crude enzyme mixture; (b) after chromatography on a column of tRNA^{Val}; (c) after chromatography first on a column of tRNA^{Val}, the unbound protein from which was then chromatographed on a column of tRNA^{Val}.

charging and recognition are more common when enzyme and tRNA are derived from widely differing sources.

In the present work, the ability of the tRNA^{Val} column to bind significant quantities of valyl-tRNA

synthetase merely lowered the yield of this enzyme collected from the column as non-bound protein, without affecting the overall degree of purification. This suggests that some degree of binding by the target enzyme to the preliminary affinity column can be tolerated.

Application to other aminoacyl-tRNA synthetases

The method described above should be applicable to the preparation of affinity columns designed for the purification of other aminoacyl-tRNA synthetases, subject to the following conditions. First, it must be possible to achieve a satisfactory degree of aminoacylation of the cognate tRNA. Some care may have to be exercised in cases where the aminoacyl-tRNA is more labile to hydrolysis than the valyl-tRNA^{Val} used in these experiments. Secondly, for the successful separation of cognate from non-cognate tRNA, it is imperative that the cognate tRNA remain aminoacylated during the oxidation reaction. Since this takes place at pH 5 and room temperature, conditions under which most aminoacyl-tRNA species have a half-life with respect to hydrolysis of several hours (Hentzen *et al.*, 1972), it seems likely that this condition will invariably be met. Thirdly, the system must be free from complications caused by enzyme-tRNA misrecognition and/or mischarging.

For the purification of an aminoacyl-tRNA synthetase from an organism from which neither tRNA nor enzyme are initially available, the following procedure is envisaged. First, a small-scale extract containing the aminoacyl-tRNA synthetase is required. Very low purity can be tolerated provided that the resulting enzyme solution is of sufficient concentration to be used in the preparative aminoacylation of tRNA. Crude tRNA in sufficient quantity to make the two affinity columns is then isolated by standard methods. At this stage, the optimum reaction conditions for aminoacylation of tRNA must be established. Preparation of the two columns can then take place. Before chromatography on the tRNA affinity columns, the enzyme must be freed from tRNA, which will compete with the column-bound tRNA for the enzyme binding sites, and ribonucleases, which may destroy the column material.

It should also be mentioned that, in principle, the separation of a synthetase from other enzymes that recognize general tRNA features can be accomplished by using any pair of specific tRNA columns, provided that one column is capable of retaining the target enzyme and the other is not. For example, we have achieved a similar purification of valyl-tRNA synthetase by using a tRNA^{Leu} column instead of the tRNA^{Val} column (though the misrecognition of tRNA^{Leu} by valyl-tRNA synthetase, discussed above, made this an unfortunate choice) as an illustration of the approach. This variation may provide a useful alternative in systems where the preparation and/or

use of the preliminary 'minus' column proves difficult.

Although the preparation of an appropriate pair of tRNA columns is much more rapid than the synthesis of a suitable aminoalkyladenylate analogue (see the preceding paper, Clarke & Knowles, 1977), there remain two disadvantages of tRNA columns. First, the lower resistance of tRNA columns to enzymic digestion necessitates at least a preliminary purification of the enzyme sample to remove ribonucleases. Secondly, the higher molecular weight of tRNA compared with the adenylate means that a tRNA column has a much lower capacity for the target enzyme than an adenylate column with comparable substitution (in mg/ml).

Problems deriving from misrecognition are relatively easily eliminated with either type of ligand. In the preceding paper (Clarke & Knowles, 1977) we found that valyl-tRNA synthetase, which showed some retardation on a leucyl-AMP column, was cleanly separated from the target enzyme by using either a two-column procedure or specific elution with substrate, and it seems reasonable to suppose that selective elution will invariably be possible in the few cases where misrecognition of a non-cognate aminoalkyladenylate occurs. Synthetase-tRNA misrecognition is more widespread, particularly in heterologous systems, although the problem can be minimized, if not eliminated, by the use of homologous tRNA.

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References

- Bartkowiak, S. & Pawelkiewicz, J. (1972) *Biochim. Biophys. Acta* **272**, 137–140
- Befort, J. J., Befort, N., Petrisant, G., Remy, P. & Ebel, J.-P. (1974) *Biochimie* **56**, 625–630
- Bonnet, J. & Ebel, J.-P. (1972) *Eur. J. Biochem.* **31**, 335–344
- Clarke, C. M. & Knowles, J. R. (1977) *Biochem. J.* **167**, 405–417
- Cramer, F., von der Haar, F. & Schlimme, E. (1968) *FEBS Lett.* **2**, 136–139
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Giegé, R., Kern, D., Ebel, J.-P., Grosjean, H., De Henau, S. & Chantrenne, H. (1974) *Eur. J. Biochem.* **45**, 351–362
- Hansske, F., Sprinzl, M. & Cramer, F. (1974) *Bioorg. Chem.* **3**, 367–376
- Hayashi, H. (1973) *J. Biochem. (Tokyo)* **74**, 203–208
- Hentzen, D., Mandel, P. & Garel, J.-P. (1972) *Biochim. Biophys. Acta* **281**, 228–232
- Joyce, C. M. & Knowles, J. R. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1278–1285
- Lam, S. S. M. & Schimmel, P. R. (1975) *Biochemistry* **14**, 2775–2780
- Lamed, R., Levin, Y. & Wilchek, M. (1973) *Biochim. Biophys. Acta* **304**, 231–235
- Nelidova, O. D. & Kisselev, L. L. (1968) *Mol. Biol. (Moscow)* **2**, 60–68
- Pachmann, U., Cronvall, E., Rigler, R., Hirsch, R., Wintermeyer, W. & Zachau, H. G. (1973) *Eur. J. Biochem.* **39**, 265–273
- RajBhandary, U. L. (1968) *J. Biol. Chem.* **243**, 556–564
- Remy, P., Birmelé, C. & Ebel, J.-P. (1972) *FEBS Lett.* **27**, 134–138
- Söll, D. & Schimmel, P. R. (1974) *Enzymes 3rd Ed.* **10**, 489–538
- von der Haar, F., Schlimme, E., Gómez-Guillen, M. & Cramer, F. (1971) *Eur. J. Biochem.* **24**, 296–302
- Wilkinson, S. & Knowles, J. R. (1974) *Biochem. J.* **139**, 391–398
- Yarus, M. & Berg, P. (1969) *J. Mol. Biol.* **42**, 171–189
- Zamecnik, P. C., Stephenson, M. L. & Scott, J. F. (1960) *Proc. Natl. Acad. Sci. U.S.A.* **46**, 811–822
- Zubay, G. (1962) *J. Mol. Biol.* **4**, 347–356