Valyl- and Phenylalanyl-tRNA Synthetase from Baker's Yeast: Recognition of Transfer RNA Results from a Multistep Process, as Indicated by Inhibition of Aminoacylation with Modified Transfer RNA†

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ABSTRACT: Selection of a cognate tRNA from a population of all tRNAs by its aminoacyl-tRNA synthetase is interpreted to be a multistep process. For the valyl-tRNA synthetase and for the phenylalanyl-tRNA synthetase, three and four steps, respectively, could be resolved. The enzyme·tRNA complex must pass through these distinct stages prior to transfer of the amino acid to the tRNA. Apart from many pieces of evidence already presented in the literature, the new results which lead to this interpretation are: (a) $tRNA^{Ile}$-A-C-C-A is readily misaminoacylated at low ionic strength by valyl- and phenylalanyl-tRNA synthetase, respectively. In both systems, $tRNA^{Ile}$-A-C-C-A exhibits almost the same K_M as the cognate tRNA. (b) With valyl-tRNA synthetase, $tRNA^{Val}$-A-C-C and $tRNA^{Val}$-A-C-C-2′dA are 200-fold less effective in inhibiting valylation of $tRNA^{Val}$-A-C-C-A than they are in inhibiting valylation of $tRNA^{Ile}$-A-C-C-A. With phenylalanyl-tRNA synthetase, the inhibition of phenylalanylation of $tRNA^{Phe}$-A-C-C-A by $tRNA^{Phe}$-A-C-C is 200-fold less effective, and by $tRNA^{Phe}$-A 360-fold less effective, than the inhibition of phenylalanylation of $tRNA^{Ile}$-A-C-C-A. (c) Whereas with $tRNA_{Y}^{Phe}$-A-C-C the inhibition of phenylalanylation of $tRNA^{Phe}$-A-C-C-A is not detectable, inhibition of phenylalanylation of $tRNA^{Ile}$-A-C-C-A is still very pronounced. (d) These inhibition data are extended and supported by the competitive binding of the various tRNAs under true equilibrium conditions. $tRNA^{Phe}$-A-C-C-A, $tRNA^{Phe}$-A-C-C, and $tRNA^{Ile}$-A-C-C-A all bind very efficiently to phenylalanyl-tRNA synthetase. If $tRNA^{Phe}$-A-C-C-A and $tRNA^{Phe}$-A-C-C are allowed to compete for binding to the enzyme, they bind according to mass law. If $tRNA^{Ile}$-A-C-C-A and $tRNA^{Phe}$-A-C-C compete for the enzyme, the binding of $tRNA^{Ile}$-A-C-C-A is almost completely suppressed, despite the fact that the binding constants for these tRNAs are not very different. Identical results are obtained for the valine system.

The specificity of aminoacylation of tRNA was originally thought to be due to specific complex formation between a specific tRNA and the corresponding aminoacyl-tRNA synthetase (Kisselev and Favorova, 1974; Loftfield, 1972; Söll and Schimmel, 1974). During recent years, however, increasing evidence has accumulated indicating that nonspecific interactions are the rule rather than the exception (Ebel et al., 1973; Bonnet and Ebel, 1975; von der Haar, 1976). Working under so called "special conditions", i.e., with highly purified tRNA and at low ionic strength not only could complex formation between nearly any given aminoacyl-tRNA synthetase and any noncognate tRNA be demonstrated but even misaminoacylation occurred to some extent (Giegè et al., 1974). From this information Ebel and his co-workers concluded that—in terms of Michaelis–Menten kinetics—specificity of aminoacylation originated from differences in V_{max} rather than from differences in K_M (Ebel et al., 1973; Bonnet and Ebel, 1975). This poses the question of how a complex between a tRNA molecule and its corresponding aminoacyl-tRNA synthetase is triggered to aminoacylate only bound cognate tRNA and how aminoacylation of bound noncognate tRNA is prevented (von der Haar, 1976). Logically this can be achieved only if during the course of aminoacylation the tRNA·aminoacyl-tRNA synthetase complex has to pass through several distinct kinetically controlled states. Recently, we observed that in the phenylalanine system from baker's yeast a catalytically incompetent complex is triggered to a catalytically competent one by the invariant adenosine positioned at the 3′ terminus of the tRNA. This conclusion was drawn from a series of inhibition studies with $tRNA^{Phe}$ modified at the 3′ terminus. Several of the tRNA molecules thus modified fortunately inhibited the reaction pathway at different stages (von der Haar and Gaertner, 1975). Subsequently, these results were confirmed in a rapid kinetic investigation (Krauss et al., 1977).

We now extend this approach to the analysis of the selection of noncognate vs. cognate tRNA. For this purpose, the inhibition of mischarging of $tRNA^{Ile}$-A-C-C-A by phenylalanyl- and valyl-tRNA synthetase caused by the respective 3′-terminal modified cognate tRNAs was investigated. These data are compared with competitive binding of these tRNAs under true equilibrium conditions.

Experimental Section

Enzymes. Phenylalanyl-tRNA synthetase (EC 6.1.1.20) of specific activity 1680 nmol/mg·min and valyl-tRNA synthetase (EC 6.1.1.9) of specific activity 399 nmol/mg·min were purified by affinity elution (von der Haar, 1973). tRNA-nucleotidyl transferase (EC 2.7.7.25) was prepared by Dr. Hans Sternbach in our laboratory according to Sternbach et al., (1971).

$tRNA^{Phe}$. Unfractionated tRNA from baker's yeast was purchased from Boehringer Mannheim (Germany). From this material $tRNA^{Phe}$-A-C-C and $tRNA^{Phe}$-A-C-C-A were purified as described (Schneider et al., 1972). Whereas $tRNA^{Phe}$-A-C-C was obtained directly from unfractionated tRNA from baker's yeast, in order to obtain $tRNA^{Phe}$-A-C-C-A the 3′-adenosine, which was absent to a degree greater than 85% in this particular preparation, had to be incorporated with the aid of tRNA-nucleotidyl transferase prior to the final Sephadex A-25 chromatography (Schneider et al., 1972). $tRNA^{Phe}$-A-C and $tRNA^{Phe}$-A were obtained from $tRNA^{Phe}$-A-C-C by one or two cycles of periodate oxidation,

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followed by lysine-catalyzed elimination and finally phosphatase treatment (Tal et al., 1972). After each reaction cycle, the $tRNA^{Phe}$ was rechromatographed on the Sephadex A-25 column as described (Schneider et al., 1972). This additional step led to $tRNA^{Phe}$-A-C or $tRNA^{Phe}$-A, respectively, which, in the presence of tRNA-nucleotidyl transferase, ATP, CTP, and phenylalanyl-tRNA synthetase accepted 1.55 nmol of phenylalanine/A_{260} unit. $tRNA^{Phe}$-A-C-C-A, $tRNA^{Phe}$-A-C-C, $tRNA^{Phe}$-A-C, and $tRNA^{Phe}$-A were shown to be more than 95% homogeneous with respect to their 3′-terminal nucleotide by the combination of enzymatic analysis and nucleoside analysis previously described (Sprinzl et al., 1972). $tRNA_{-Y}^{Phe}$-A-C-C was obtained by treatment of $tRNA^{Phe}$-A-C-C with 0.1 M ammonium formate buffer, pH 2.9, for 8 h at 37 °C. The material was chromatographed on benzoylated DEAE[1]-cellulose as described (Thiebe and Zachau, 1968). It accepted 1.4 nmol of phenylalanine/A_{260} unit. [^{14}C]ATP was incorporated into $tRNA^{Phe}$-A-C-C and [^{14}C]CTP was incorporated into $tRNA^{Phe}$-A-C similarly to the incorporation of unlabeled ATP and CTP (Schneider et al., 1972).

$tRNA^{Val}$ and $tRNA^{Ile}$. $tRNA^{Val}$-A-C-C-A, $tRNA^{Val}$-A-C-C, and $tRNA^{Ile}$-A-C-C-A were prepared using basically the same procedure as for the corresponding $tRNA^{Phe}$, namely, BD-cellulose, single-step liquid–liquid partition, and Sephadex A-25 chromatography (Schneider et al., 1972). Pure isoacceptors were isolated by the method of salting out on Sepharose 4B (Holmes et al., 1975) as described (von der Haar and Cramer, 1978). The experiments described were performed with $tRNA_1^{Val}$ and $tRNA_2^{Ile}$, the main isoacceptors. $tRNA^{Val}$-A-C-C-2′dA was derived from $tRNA^{Val}$-A-C-C by incorporation of 2′-dAMP with tRNA-nucleotidyl transferase as described (Cramer et al., 1975). Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). ^{14}C-labeled amino acids of Stanstar grade were obtained from Schwarz Bioresearch (Orangeburg, N.Y.). [^{14}C]ATP and [^{14}C]CTP were purchased from Buchler-Amersham (Braunschweig, Germany). All other salts and reagents were of the highest purity commercially available.

Aminoacylation. The assay mixture consisted of a 100-μL volume containing 10 mM Tris-HCl buffer (pH 9.0), 8 mM $MgSO_4$, 0.5 mM ATP, and 0.06 mM ^{14}C-labeled amino acid. Because the amino acid was added in 10 mM HCl solution, the actual pH during aminoacylation was 8.5. The concentrations of substrate tRNA and of the inhibiting tRNA are specified in the text. The assay was performed at 37 °C. After 1, 2, 3, and 5 min, 10-μL aliquots were withdrawn from the assay and prepared for liquid scintillation counting as described (Schlimme et al., 1969).

Michaelis–Menten constants were derived from double-reciprocal plots of data obtained from the aminoacylation assay.

Fifty percent inhibition was determined by performing the aminoacylation assay with various substrates at constant concentrations specified in the tables in the presence and absence of increasing amounts of inhibitor. The enzyme concentration was chosen such that the values were linear over 3 min. The initial velocities thus obtained were normalized and plotted in a semilogarithmic plot (Figure 1).

Competitive Binding of tRNA under True Equilibrium Conditions. The method of Hummel and Dreyer (1962) was chosen to follow binding of tRNA to aminoacyl-tRNA synthetases. A 65 × 1.8 cm column filled with Sephadex G-200 was equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 8 mM $MgSO_4$. tRNA was added as specified in the legends to the figures. Fractions of 1.9 mL were collected. Changes in absorption at 280 and 260 nm, respectively, were determined against absorption in the equilibrating buffer. Competitive binding was followed by addition into the buffer of two tRNA species, one of which was radioactively labeled. In this case, in addition to changes in absorption, 50-μL aliquots of each fraction were spotted onto Whatman 3MM filter disks. The filters were dried and radioactivity was determined in a Tri-Carb liquid scintillation counter using a POP/POPOP scintimix cocktail.

TABLE I: Michaelis–Menten Kinetic Data.

enzyme	substrate	K_M (μM)	$V_{max,rel}$	$V_{max,cog}/V_{max,ncog}$
Phe-tRNA synthetase	$tRNA^{Phe}$-A-C-C-A	2.6	100	60
	$tRNA^{Ile}$-A-C-C-A	4.6	1.6	
Val-tRNA synthetase	$tRNA^{Val}$-A-C-C-A	5.0	100	160
	$tRNA^{Ile}$-A-C-C-A	3.2	0.6	

Results

Mischarging Conditions. By systematic variation of pH, ionic strength, Mg^{2+}, and ATP concentration, we ascertained that $tRNA^{Ile}$-A-C-C-A was very effectively misaminoacylated by valyl- as well as by phenylalanyl-tRNA synthetase under the assay conditions given under the Experimental Section. It should be noted that this misaminoacylation was achieved in the absence of any organic solvent, which has often been used in the misaminoacylation studies reported earlier (Ebel et al., 1973). The presence of 10–20% dimethyl sulfoxide in our assay also significantly enhanced the velocity of misaminoacylation. However, we avoided the presence of organic solvents in the assay, since it is not known whether they influence primarily the tRNA or the enzyme (Ebel et al., 1973).

Michaelis–Menten Constants of Cognate and Noncognate tRNAs. In the phenylalanine and valine systems, the K_M for $tRNA^{Ile}$-A-C-C-A was, within experimental error, almost identical to the K_M for the cognate $tRNA^{Phe}$-A-C-C-A and $tRNA^{Val}$-A-C-C-A, respectively (Table I). A considerable difference, however, was found between the V_{max} of the cognate tRNA and the noncognate $tRNA^{Ile}$-A-C-C-A. In the phenylalanine system, $V_{max,noncognate}$ was reduced to 1.6%, and in the valine system it was reduced to 0.6%. It was ascertained that no aminoacylation of $tRNA^{Ile}$A-C-C-A occurred with either enzyme in the presence of [^{14}C]isoleucine. This indicated that both enzyme preparations were completely free of isoleucyl-tRNA synthetase.' Therefore, aminoacylation of $tRNA^{Ile}$A-C-C-A was due to phenylalanyl- and valyl-tRNA synthetase, respectively; a conclusion further supported by the inhibition studies described below.

Inhibition of Aminoacylation of $tRNA^{Val}$-A-C-C-A and of $tRNA^{Ile}$-A-C-C-A by $tRNA^{Val}$-A-C-C-2′dA and by $tRNA^{Val}$-A-C-C. In view of the earlier results (von der Haar and Gaertner, 1975) and because $tRNA^{Val}$-A-C-C-2′dA did not show substantial inhibition of aminoacylation of $tRNA^{Val}$-A-C-C-A (von der Haar and Cramer, 1978), we

[1] Abbreviations used are: $tRNA^{Xxx}$ or $tRNA^{Xxx}$-A-C-C-A, tRNA specific for amino acid Xxx with complete 3′-terminal nucleotide sequence; $tRNA^{Xxx}$-A-C-C, $tRNA^{Xxx}$-A-C, etc., tRNAs lacking 3′-terminal nucleotides; $tRNA^{Xxx}$-A-C-C-3′dA, tRNA with 3′-deoxyadenosine at its 3′ terminus instead of adenosine (analogously F = formycin = 8-aza-9-deazaadenosine, A_{oxi} = periodate-oxidized adenosine bearing a dialdehyde moiety at the ribose, $A_{oxi\text{-}red}$ = periodate-oxidized and subsequently borohydride-reduced adenosine, F_{oxi} and $F_{oxi\text{-}red}$ as terminal nucleoside); $tRNA_{-Y}^{Phe}$-A-C-C, $tRNA^{Phe}$ lacking both the 3′-terminal AMP and the Y base in the anticodon loop; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

TABLE II: Fifty Percent Inhibition Data.

enzyme	substrate	substrate concn (μM)	inhibitor	50% inhibition concn (μM)
Phe-tRNA synthetase	$tRNA^{Phe}$-A-C-C-A	6.6	$tRNA^{Phe}$-A-C-C	39 ± 2
	$tRNA^{Phe}$-A-C-C-A	6.6	$tRNA^{Phe}$-A	115 ± 5
	$tRNA^{Phe}$-A-C-C-A	2.0	$tRNA^{Phe}_{-Y}$-A-C-C	not detect.
Phe-tRNA synthetase	$tRNA^{Ile}$-A-C-C-A	4.0	$tRNA^{Phe}$-A-C-C	0.25 ± 0.04
	$tRNA^{Ile}$-A-C-C-A	6.4	$tRNA^{Phe}$-A-C	0.26 ± 0.04
	$tRNA^{Ile}$-A-C-C-A	6.4	$tRNA^{Phe}$-A	0.32 ± 0.04
	$tRNA^{Ile}$-A-C-C-A	6.4	$tRNA^{Phe}_{-Y}$-A-C-C	0.80 ± 0.03
Val-tRNA synthetase	$tRNA^{Val}$-A-C-C-A	2.5	$tRNA^{Val}$-A-C-C-2′dA	39 ± 2
	$tRNA^{Ile}$-A-C-C-A	8.4	$tRNA^{Val}$-A-C-C-2′dA	0.22 ± 0.04
	$tRNA^{Ile}$-A-C-C-A	8.4	$tRNA^{Val}$-A-C-C	0.16 ± 0.04

expected a complicated kinetic scheme for the aminoacylation. In such a system, a Michaelis-Menten kinetic investigation does not yield interpretable results. Therefore, we restricted ourselves to the determination of that concentration of inhibitory tRNA which caused 50% inhibition of aminoacylation of a particular substrate. The type of analysis is demonstrated in Figure 1 and the data derived are summarized in Table II. Fifty percent inhibition with $tRNA^{Val}$-A-C-C-2′dA was obtained at 39 μM concentration at a substrate concentration of 2.5 μM for $tRNA^{Val}$-A-C-C-A. To achieve the same inhibition for the noncognate $tRNA^{He}$-A-C-C-A, a 200-fold lower concentration was necessary even if the substrate concentration was increased mor more than threefold. $tRNA^{Val}$-A-C-C showed an identical behavior to $tRNA^{Val}$-A-C-C-2′dA.

Inhibition of Aminoacylation of $tRNA^{Phe}$-A-C-C-A and of $tRNA^{Ile}$-A-C-C-A by Modified $tRNA^{Phe}$. $tRNA^{Phe}$-A-C-C showed the same behavior in the phenylalanine system as $tRNA^{Val}$-A-C-C-2′dA did in the valine system (Table II). The inhibitory ability of modified $tRNA^{Phe}$ was substantially decreased if both cytidine monophosphates of the 3′ terminus were absent, as in $tRNA^{Phe}$-A. Another position which is easily modified in $tRNA^{Phe}$ is the highly modified base next to the anticodon. This base can be removed by acid treatment (Thiebe and Zachau, 1968), leaving the phosphodiester backbone intact. We therefore included $tRNA^{Phe}_{-Y}$-A-C-C in our investigation in order to obtain information about the influence of the anticodon loop on the aminoacylation reaction. Even at a very low substrate concentration (2 μM) of $tRNA^{Phe}$-A-C-C-A, virtually no inhibition was detectable.

The aminoacylation of $tRNA^{Ile}$-A-C-C-A by phenylalanyl-tRNA synthetase was inhibited by a 200-fold lower concentration of $tRNA^{Phe}$-A-C-C than was needed for the inhibition of cognate $tRNA^{Phe}$-A-C-C-A. $tRNA^{Phe}$-A inhibited aminoacylation of $tRNA^{Ile}$-A-C-C-A at a concentration 360-fold lower than for $tRNA^{Phe}$-A-C-C-A. Whereas $tRNA^{Phe}_{-Y}$-A-C-C did not detectably inhibit the aminoacylation of $tRNA^{Phe}$-A-C-C-A, its ability to inhibit the mischarging of $tRNA^{Ile}$-A-C-C-A was only threefold less than that of $tRNA^{Phe}$-A-C-C (Table II).

Competitive Binding of tRNA to Aminoacyl-tRNA Synthetases. The experiments given in Figures 2 and 3, monitoring changes in UV absorption, allow one to determine the total amount of tRNA bound to the enzyme, irrespective of whether it is cognate or noncognate. The changes in the level of radioactivity, however, account only for that species which is radioactively tagged. Hence, from the known specific activity of the labeled tRNA, its amount in a given fraction can be quantitized. The amount of unlabeled tRNA present can be calculated by subtraction of labeled tRNA from total tRNA.

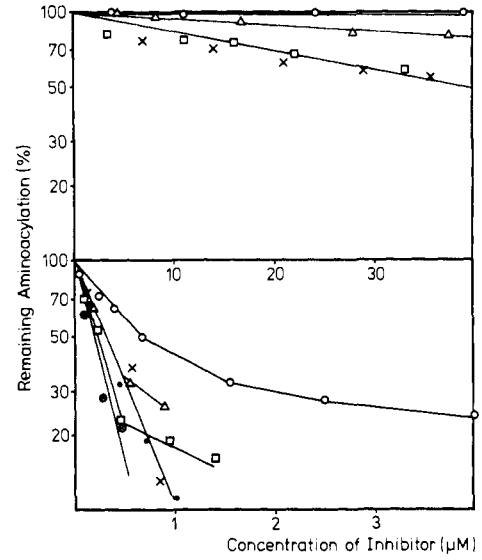


FIGURE 1: Determination of 50% inhibition. Data were obtained from the aminoacylation assay with the following enzymes and tRNAs as described under the Experimental Section: (Top) $tRNA^{Phe}$-A-C-C-A, phenylalanyl-tRNA synthetase, $tRNA^{Phe}$-A-C-C (x); $tRNA^{Phe}$-A-C-C-A, phenylalanyl-tRNA synthetase, $tRNA^{Phe}$-A (△); $tRNA^{Phe}$-A-C-C-A, phenylalanyl-tRNA synthetase, $tRNA^{Phe}_{-Y}$-A-C-C (○); $tRNA^{Val}$-A-C-C-A, valyl-tRNA synthetase, $tRNA^{Val}$-A-C-C-2′dA (□). (Bottom) $tRNA^{Ile}$-A-C-C-A, phenylalanyl-tRNA synthetase, $tRNA^{Phe}$-A-C-C (x); $tRNA^{Ile}$-A-C-C-A, phenylalanyl-tRNA synthetase, $tRNA^{Phe}$-A-C (●); $tRNA^{Ile}$-A-C-C-A, phenylalanyl-tRNA synthetase, $tRNA^{Phe}$-A (△); $tRNA^{Ile}$-A-C-C-A, valyl-tRNA synthetase, $tRNA^{Phe}_{-Y}$-A-C-C (○); $tRNA^{Ile}$-A-C-C-A, valyl-tRNA synthetase, $tRNA^{Val}$-A-C-C-2′dA (□); $tRNA^{Ile}$-A-C-C-A, valyl-tRNA synthetase, $tRNA^{Val}$-A-C-C (⊗).

Under the conditions chosen, $tRNA^{Phe}$-A-C-C-A as well as $tRNA^{Ile}$-A-C-C-A bind efficiently to the phenylalanyl-tRNA synthetase (Figure 2A,B). Since the extinction coefficient of the individual components are known, the stoichiometry of the complex can be calculated from the ratio A_{260}/A_{280} in the peak. The stoichiometries are 1.05 mol of $tRNA^{Ile}$-A-C-C-A and 1.80 mol of $tRNA^{Phe}$-A-C-C-A per enzyme molecule. The same values calculated from the integrated absorption values of the trough are 0.95 and 1.60, respectively. This is in agreement with the earlier observation that phenylalanyl-tRNA synthetase from baker's yeast is able to bind only one noncognate tRNA in contrast to two cognate $tRNA^{Phe}$ (Krauss et al., 1976).

In competitive binding (Figure 2C), $tRNA^{Phe}$-A-C-C and $tRNA^{Phe}$-A-C-C-A bind roughly proportionally to their relative concentrations in the equilibrating solution. This is to be expected in light of the nearly identical dissociation constants (Krauss et al., 1976, 1977); it is, however, in contrast to the lack

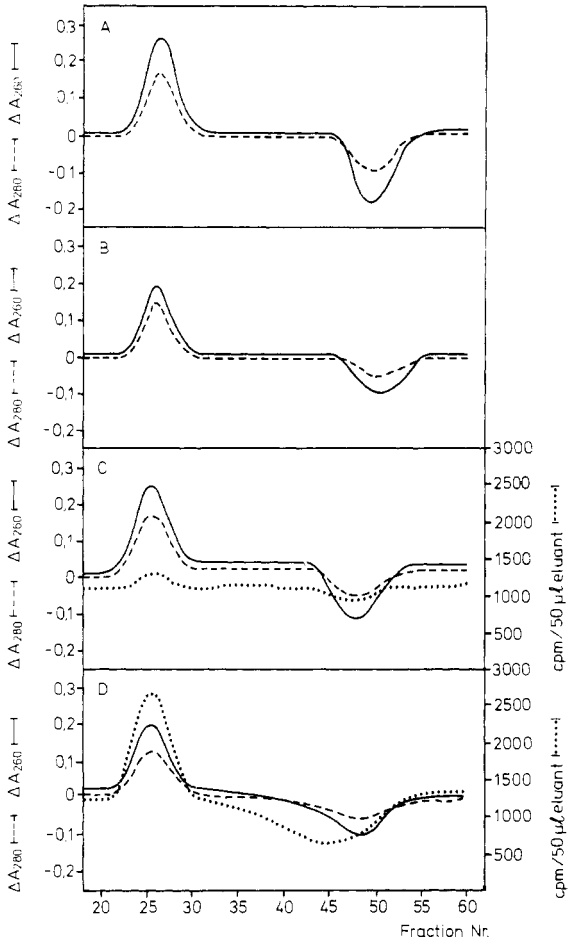


FIGURE 2: Competitive binding of tRNA to phenylalanyl-tRNA synthetase. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 8 mM $MgSO_4$ in addition to 2.55 μM $tRNA^{Phe}$-A-C-C-A (A), 2.55 μM $tRNA^{Ile}$-A-C-C-A (B), 2.04 μM $tRNA^{Phe}$-A-C-C and 0.207 μM $tRNA^{Phe}$-A-C-C-[^{14}C]A (C), and 2.21 μM $tRNA^{Ile}$-A-C-C-A and 0.172 μM $tRNA^{Phe}$-A-C-[^{14}C]C (D). In each case, 1.5 nmol of phenylalanyl-tRNA synthetase was applied to the column in 0.5 mL of buffer.

of inhibition of aminoacylation of $tRNA^{Phe}$-A-C-C-A by $tRNA^{Phe}$-A-C-C (von der Haar and Gaertner, 1975).

In competitive binding of $tRNA^{Ile}$-A-C-C-A vs. $tRNA^{Phe}$-A-C-C, the cognate $tRNA^{Phe}$-A-C-C is strongly selected against the noncognate $tRNA^{Ile}$-A-C-C-A (Figure 2D). The small trough in absorption vs. the broad trough in radioactivity indicates that $tRNA^{Ile}$-A-C-C-A is primarily bound, since enzyme is in excess over cognate $tRNA^{Phe}$-A-C-C. During the passage along the column, however, $tRNA^{Phe}$-A-C-C is selected against the excess of $tRNA^{Ile}$-A-C-C-A. From the integrated radioactivity in the peak, a ratio of 1.5 mol of $tRNA^{Phe}$-A-C-C formed per enzyme molecule is calculated. The result is in agreement with the inhibition data given above; it is, however, not expected in the light of the fact that K_{dissoc} is almost identical for cognate and noncognate tRNA under the conditions applied (Figure 2b; von der Haar, 1976; Krauss et al., 1977).

Regarding the competitive binding in the valine system, identical results as for the phenylalanine system are found (Figure 3A,B). From the integrated radioactivity, a stoichiometry of 1.15 mol of tRNA complexed to one enzyme molecule is determined (Figure 3B), the equivalent value as calculated from the absorption in the trough (Figure 2A) is 0.82.

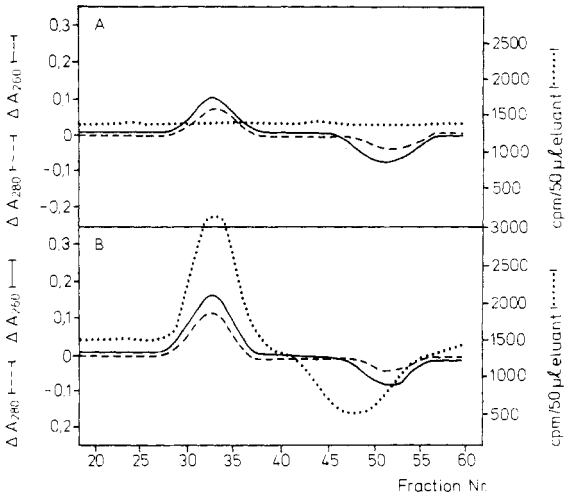


FIGURE 3: Competitive binding of tRNA to valyl-tRNA synthetase. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 8 mM $MgSO_4$ in addition to 2.04 μM $tRNA^{Val}$-A-C-C and 0.17 μM $tRNA^{Val}$-A-C-C-[^{14}C]A (A) and 2.04 μM $tRNA^{Ile}$-A-C-C-A and 0.17 μM $tRNA^{Val}$-A-C-C-[^{14}C]A (B). 1.97 and 2.95 nmol of enzyme were applied to A and B, respectively.

Discussion

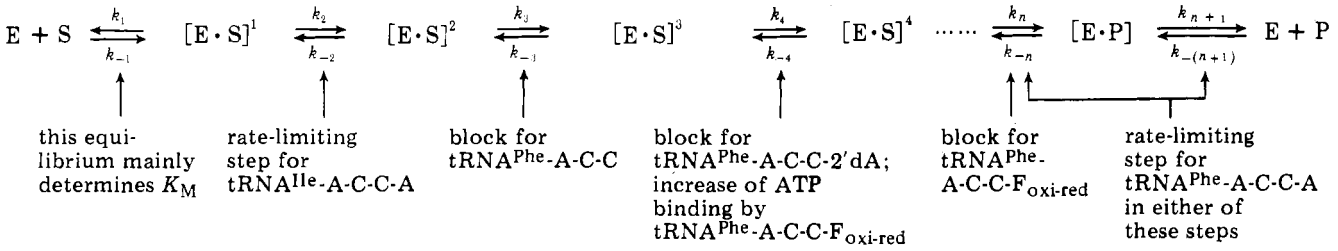
It is a well-established fact that individual aminoacyl-tRNA synthetases can catalyse the aminoacylation of cognate and noncognate tRNA (Giegè et al., 1974; Roe et al., 1973). Hence, specificity of aminoacylation of the cognate tRNA in vivo must have the characteristics of a dynamic selection process rather than those of an interaction solely determined by thermodynamic factors. Insight into such a selection process can be gained from competition experiments. Since the products of such a selection process—aminoacylated cognate tRNA vs. aminoacylated noncognate tRNA—are difficult to distinguish, one is restricted to inhibition studies to elucidate the dynamics of the process.

For competition studies under true equilibrium conditions, physicochemical studies as applied by Krauss et al. (1976, 1977) are difficult to perform, since the same signal is influenced by both cognate and noncognate tRNA. Hence, we decided to label one component with a radioisotope and follow the fate of it in comparison to the total UV extinction.

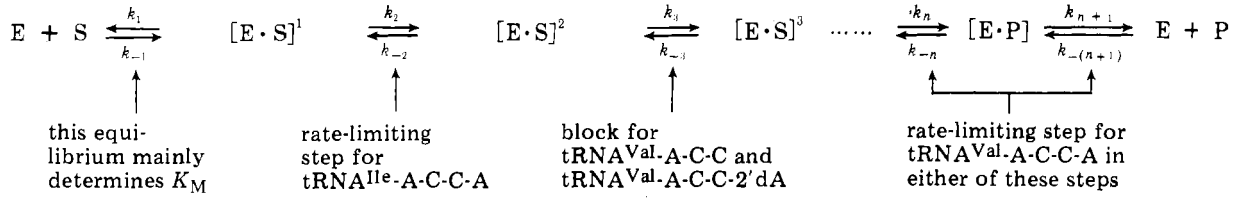
Kinetic Course of Aminoacylation in the Phenylalanine System. An attempt to rationalize the inhibition data for aminoacylation of $tRNA^{Phe}$-A-C-C-A and $tRNA^{Ile}$-A-C-C-A in the phenylalanine system is outlined in Scheme I. This scheme is derived assuming that the cognate $tRNA^{Phe}$ induces several conformational changes in order to allow the reaction to proceed. This type of analysis is an extrapolation of the work concerned with the role of the invariant 3′-terminal adenosine in the dynamic recognition of tRNA (von der Haar and Gaertner, 1975; Krauss et al., 1977; von der Haar and Cramer, 1978).

Biomolecular Association between tRNA and Enzyme in Relation to K_M. The Michaelis–Menten constant K_M is a complex value determined by the ratio of several rate constants. Nevertheless, we attribute the numerical identity of K_M for cognate and noncognate tRNA primarily to an identity of k_{+1} and k_{-1} for these different substrates (Scheme I) for the following reasons: (1) For the phenylalanine system from baker's yeast as well as for the isoleucine system from *E. coli*, the K_M for a number of noncognate homologous and heterologous tRNAs was found to differ by less than a factor of 10 (Roe et al., 1973). This difference in K_M is much smaller than the

SCHEME I: Kinetic Course in the Phenylalanine System.



SCHEME II: Kinetic Course in the Valine System.



difference in the equilibrium constants determined for noncognate and cognate tRNA–enzyme interactions in the isoleucine system from *E. coli* and the valine system from baker's yeast (Lam and Schimmel, 1975). Identical values of K_M were also found for tRNA^{Phe}-A-C-C-F and tRNA^{Phe}-A-C-C-A in the phenylalanine system, in which the V_{max} for these two substrates differed by a factor of more than 25 (von der Haar and Gaertner, 1975). (2) k_{+1} has been shown to be nearly diffusion controlled for cognate and noncognate tRNAs with the phenylalanine system (Krauss et al., 1973, 1976) for which reason this step cannot be very specific. Identical K_M values in all these cases would then arise from differences in k_{-1} balanced by an opposing effect in later rate constants. This we feel is inherently unlikely to occur for so many tRNAs. (3) In the phenylalanine system from baker's yeast, the dissociation constants for noncognate and for cognate tRNAs are of the same order of magnitude (μM), which is nearly identical to K_M (Krauss et al., 1976). Hence, it seems probable that the bimolecular complex formation between tRNA and enzyme is a rapid equilibrium which is almost identical for cognate and noncognate tRNA (Scheme I).

Conformational Transitions during the Course of Aminoacylation. One conformational change giving rise to the selection of tRNA^{Phe}-A-C-C-A vs. tRNA^{Phe}-A-C-C (k_3/k_{-3} in Scheme I) has been firmly established (von der Haar and Gaertner, 1975; Krauss et al., 1977). Are there analogous conformational transitions for the selection of tRNA^{Phe}-A-C-C vs. tRNA^{Ile}-A-C-C-A? tRNA^{Phe}-A-C-C, under true equilibrium conditions (Figure 2C,D) as well as during aminoacylation (Table II), is much more efficient in competing with tRNA^{Ile}-A-C-C-A for phenylalanyl-tRNA synthetase than would be expected according to mass law if we take into account the identical K_M (Table I) and the nearly identical K_{dissoc} (Krauss et al., 1976). This discrepancy is most easily explained assuming a conformational transition induced solely by tRNA^{Phe}-A-C-C, which, hence, disturbes the equilibrium between phenylalanyl-tRNA synthetase, tRNA^{Ile}-A-C-C-A, and tRNA^{Phe}-A-C-C from that expected according to their individual binding constants (k_2/k_{-2} in Scheme I). That this transition has so far escaped detection by the rapid kinetic investigations may be due to the fact that it is either too fast to be resolved or, alternatively, that it is not accompanied by an alteration in the signal used for detection in these investigations.

The structural element in tRNA^{Phe} responsible for induction of this conformational transition and the way it actually functions is not known. It is definitively not the 3′-terminal adenosine and is not the Y base from the anticodon loop, since tRNA$^{Phe}_{Y}$-A-C-C lacking both these elements is able to inhibit misaminoacylation of tRNA^{Ile}-A-C-C-A (Table II). In this respect, it is of interest, however, that for the methionyl-tRNA synthetase from *E. coli* a conformational transition induced by base no. 36 in the anticodon seems very likely (Stern and Schulman, 1977; Schulman and Pelka, 1977).

A conformational change as described above may also explain the fact that phenylalanyl-tRNA synthetase binds only one noncognate tRNA in contrast to two cognate tRNAs. It was originally speculated that one noncognate tRNA might mask both binding sites simultaneously (Krauss et al., 1976). An alternative speculation may be that the second binding site can be occupied by a tRNA only if the conformational transition (k_2/k_{-2} in Scheme I) has taken place. Such a speculation is not unlikely in view of the interaction between the two binding sites of isoleucyl-tRNA synthetase from baker's yeast described earlier (von der Haar and Cramer, 1978). This interplay of the binding sites is possibly responsible for preventing binding of tRNA^{Ile} to the enzyme in the presence of tRNA^{Phe} (Figure 2D). The existence of such an interbinding site response, which would also imply a two-site binding of tRNA^{Val} to valyl-tRNA synthetase (see below), provides a challenge for the elucidation of the exact nature of this interplay.

By an analogous analysis, we can resolve the phenylalanine system one step further. As shown earlier, tRNA^{Phe}-A-C-C-2′dA and tRNA^{Phe}-A-C-C-F$_{oxi-red}$ are both strong competitive inhibitors in the aminoacylation reaction. They differ, however, in that, on ATP/PP exchange with the tRNA^{Phe}-A-C-C-F$_{oxi-red}$·phenylalanyl-tRNA synthetase complex, the K_M for ATP is reduced sevenfold as compared to ATP/PP exchange with the free enzyme or with the tRNA^{Phe}-A-C-C-2′dA·phenylalanyl-tRNA synthetase complex (von der Haar and Gaertner, 1975). Thus, both these complexes must be different and for their interconversion a distinct monomolecular step must consequently be postulated (k_4/k_{-4}, in Scheme I).

Thus, in the phenylalanine system we can resolve the course of events into one bimolecular step followed by three monomolecular steps. There must exist at least one, but more probably n, further steps prior to product formation. The final step will then be the release of product. The rate-limiting step for the cognate tRNA^{Phe}-A-C-C-A may be in the series of n steps or in the release of product (Scheme I).

Kinetic Course of Aminoacylation in the Valine System.

With respect to the K_M and the following monomolecular steps k_2/k_{-2} and k_3/k_{-3}, the situation in the valine system is completely analogous to the phenylalanine system (Scheme II). Since in this system the induction of the monomolecular step k_3/k_{-3} is a function of the accepting 2'-OH of the 3'-terminal adenosine (von der Haar and Cramer, 1978), we have *no information* as to whether a step k_4/k_{-4} analogous to the phenylalanine system exists or not. Regarding product formation and its release, the situation is again analogous to the phenylalanine system.

Conclusion. The data presented in this paper indicate that recognition of tRNA is not a simple process but a rather complicated sequence of events. In particular, the view that an individual tRNA possesses a unique recognition site different from the recognition sites of all the other tRNAs must be revised. Much of the work on tRNA–enzyme interaction, however, has been directed by this view. From the data given here, we would be inclined to agree with Loftfield who stated that the search for a "recognition site" was a search for a "will-o'-the-wisp" (Loftfield, 1972).

Obviously, the similarity of all tRNAs, a prerequisite for proper protein biosynthesis in the ribosomal machinery, does not permit the construction of recognition sites which are sufficiently different to discriminate between individual tRNAs by forming thermodynamically stable complexes solely with the cognate tRNA. Instead, recognition of an individual tRNA is achieved by passing through a cataract of several steps. The error fraction, which may be as high as 0.016 as shown by the V_{max} of phenylalanylation of pure $tRNA^{Ile}$-A-C-C-A as compared to $tRNA^{Phe}$-A-C-C-A in the mischarging assay, is very much reduced in the presence of only minor amounts of the cognate $tRNA^{Phe}$-A-C-C-A. In this instance, the cognate tRNA competes with the noncognate tRNA in several steps. As a result, the overall error fraction is the product of the error fraction of all the steps. The driving force for the process results from triggering events which can obviously be better—if not exclusively—performed by the cognate than by the noncognate substrate.

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References

Bonnet, J., and Ebel, J. P. (1975), *Eur. J. Biochem. 58,* 193–201.

Cramer, F., Faulhammer, H., von der Haar, F., Sprinzl, M., and Sternbach, H. (1975), *FEBS Lett. 56,* 212–214.

Ebel, J. P., Giegè, R., Bonnet, J., Kern, D., Befort, N., Bollack, C., Fasiolo, F., Gangloff, J., and Dirheimer, G. (1973), *Biochimie 55,* 547–557.

Giegè, R., Kern, D., Ebel, J. P., Grosjean, H., DeHenau, S., and Chantrenne, H. (1974), *Eur. J. Biochem. 45,* 351–362.

Holmes, W. M., Hurd, R. E., Reid, B. R., Rimerman, R. A., and Hatfield, G. W. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1068–1071.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530–532.

Kan, L. S., Ts'o, P. O. P., von der Haar, F., Sprinzl, M., and Cramer, F. (1975), *Biochemistry 14,* 3278–3291.

Kisselev, L. L., and Favorova, O. O. (1974), *Adv. Enzymol. 40,* 141–238.

Krauss, G., Römer, R., Riesner, D., and Maass, G. (1973), *FEBS Lett. 30,* 6–10.

Krauss, G., Riesner, D., and Maass, G. (1976), *Eur. J. Biochem. 68,* 81–93.

Krauss, G., Riesner, D., and Maass, G. (1977), *Nucleic Acids Res. 4,* 2253–2262.

Lam, S. S. M., and Schimmel, P. R. (1975), *Biochemistry 14,* 2775–2780.

Loftfield, R. B. (1972), *Prog. Nucleic Acid Res. Mol. Biol. 12,* 87–128.

Roe, B., Sirover, M., and Dudock, B. (1973), *Biochemistry 12,* 4146–4154.

Schlimme, E., von der Haar, F., and Cramer, F. (1969), *Z. Naturforsch. B 24,* 631–637.

Schneider, D., Solfert, R., and von der Haar, F. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353,* 1330–1336.

Schulman, L. H., and Pelka, H. (1977), *Biochemistry 16,* 4256–4265.

Söll, D., and Schimmel, P. R. (1974), *Enzymes, 3rd Ed. 10,* 489–538.

Sprinzl, M., von der Haar, F., Schlimme, E., Sternbach, H., and Cramer, F. (1972), *Eur. J. Biochem. 25,* 262–266.

Stern, L., and Schulman, L. H. (1977), *J. Biol. Chem. 252,* 6403–6408.

Sternbach, H., von der Haar, F., Schlimme, E., Gaertner, E., and Cramer, F. (1971), *Eur. J. Biochem. 22,* 166–172.

Tal, J., Deutscher, M. P., and Littauer, U. Z. (1972), *Eur. J. Biochem. 28,* 478–491.

Thiebe, R., and Zachau, H. G. (1968), *Eur. J. Biochem. 5,* 546–555.

von der Haar, F. (1973), *Eur. J. Biochem. 34,* 84–90.

von der Haar, F. (1976), *Hoppe-Seyler's Z. Physiol. Chem. 357,* 819–823.

von der Haar, F., and Cramer, F. (1978), *Biochemistry 17* (in press).

von der Haar, F., and Gaertner, E. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1378–1382.