Seryl-, Threonyl-, Valyl-, and Isoleucyl-tRNA Synthetases from Baker's Yeast: Role of the 3′-Terminal Adenosine in the Dynamic Recognition of tRNA†

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ABSTRACT: The interactions of seryl-, threonyl-, valyl-, and isoleucyl-tRNA synthetases with their respective tRNAs modified at the 3′-terminal adenosine have been investigated with respect to their substrate or inhibitor properties. $tRNA^{Ser}$-C-C-3′dA, $tRNA^{Thr}$-C-C-3′dA, and $tRNA^{Val}$-C-C-2′dA (all lacking the OH which accepts the respective amino acid) are only weak inhibitors of their corresponding synthetases. All other modified tRNA-C-C-Ns tested also showed only very weak inhibition with regard to these three synthetases. In the isoleucyl system, $tRNA^{Ile}$-C-C is a very weak inhibitor; $tRNA^{Ile}$-C-C-2′dA is a competitive and $tRNA^{Ile}$-C-C-A_{oxi}, $tRNA^{Ile}$-C-C-$A_{oxi\text{-}red}$, and $tRNA^{Ile}$-C-C-$F_{oxi\text{-}red}$ are noncompetitive inhibitors. $tRNA^{Ile}$-C-C-3′dA is a substrate and shows potent substrate inhibition which is not observed with the native substrate $tRNA^{Ile}$-C-C-A. This substrate inhibition is suppressed by the presence of low concentrations of $tRNA^{Ile}$-C-C in the assay. We conclude that in all the systems investigated a conformational change in the enzyme is induced by the 3′-terminal adenosine. This adenosine is the only group involved in the esterification with the amino acid; hence we would like to call this phenomenon "reacting site triggering" (von der Haar, F., & Gaertner, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 1378–1382). In the seryl, threonyl, and valyl systems the reacting site triggering must be intimately related to the amino acid accepting OH. In contrast the triggering in the isoleucyl system is related to the nonaccepting 3′OH. It is further concluded that the isoleucyl-tRNA synthetase possesses two binding sites for $tRNA^{Ile}$-C-C-A per single chain of molecular weight 115 000. Binding of $tRNA^{Ile}$ to, and reactivity in, each binding site is related to the reacting site triggering by the nonaccepting 3′OH of the 3′-terminal adenosine; isoleucyl transfer to $tRNA^{Ile}$-C-C-A is performed only at one binding site at a time. Finally, the conformational change induced by the nonaccepting 3′OH of the 3′-terminal adenosine is a prerequisite for specific isoleucylation of $tRNA^{Ile}$-C-C-A.

During protein biosynthesis the amino acids from the cell pool are activated by aminoacyl-tRNA synthetases (eq 1) and subsequently transferred to tRNA (eq 2).

$$E^{Xxx} + ATP + aa^{Xxx} \rightarrow [E^{Xxx} \cdot aa^{Xxx}\text{–}AMP] + PP \quad (1)$$

$$[E^{Xxx} \cdot aa^{Xxx}\text{–}AMP] + tRNA \rightarrow aa^{Xxx}\text{–}tRNA^{Xxx} + AMP + E^{Xxx} \quad (2)$$

In the ribosomal machinery the incorporation of an individual amino acid is determined by the interaction of the codon of the messenger RNA with the anticodon of the tRNA, and so the reactions expressed in eq 1 and 2 have to be highly specific. In recent years progress has been made toward elucidating those factors which determine the specificity of aminoacyl-tRNA synthetases for their respective amino acids (Fersht & Kaethner, 1976; von der Haar & Cramer, 1976; von der Haar, 1976a). The specificity of the interaction between the synthetases and tRNA, however, has remained obscure.

In an earlier report we came to the conclusion that the specificity of interaction of $tRNA^{Phe}$ with phenylalanyl-tRNA synthetase from baker's yeast could be explained by a kinetic scheme involving at least two steps (eq 3) (von der Haar & Gaertner, 1975).

$$tRNA^{Phe}\text{-C-C-A} + E^{Phe} \rightleftharpoons [E^{Phe} \cdot tRNA^{Phe}]^1 \rightleftharpoons [E^{Phe} \cdot tRNA^{Phe}]^2 \quad (3)$$

A rapid bimolecular association, most probably not very specific since it is predominantly diffusion controlled (Krauss et al., 1976), is followed by a monomolecular conformational transition converting a catalytically incompetent into a catalytically competent complex. From investigations with $tRNA^{Phe}$ modified at the 3′-terminal adenosine it was concluded that this particular conformational change is triggered by the invariant 3′-terminal adenosine (von der Haar & Gaertner, 1975).

Rapid kinetic investigations have now confirmed these conclusions. In the seryl (Riegler et al., 1976) as well as in the phenylalanyl system (Krauss et al., 1976) (both from baker's yeast) it was shown that the bimolecular association is followed by a conformational change induced by the cognate $tRNA^{Ser}$ and $tRNA^{Phe}$, respectively. Because the bimolecular association was considered to be rather unspecific, it was designated as the scanning step. This is followed by the specific conformational change designated as the identification step (Riegler et al., 1976). Recently it was demonstrated that $tRNA^{Phe}$-C-C[1] lacking the 3′-terminal AMP was unable to induce this conformational change, thus confirming our conclusion that it was indeed triggered by the invariant 3′-terminal AMP (Krauss et al., 1977). It was also shown (Krauss et al., 1977) that phenylalanyl adenylate (eq 1) directly influences the binding of $tRNA^{Phe}$ (eq 2) and vice versa, a conclusion origi-

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[1] Abbreviations used: tRNA-C-C-A or tRNA, native transfer RNA; tRNA-C-C, tRNA lacking the 3′-terminal AMP; tRNA-C-C-F, tRNA-C-C-2′dA, tRNA-C-C-3′dA, and tRNA-C-C-3′NH_2A, tRNA with 3′-terminal formycin (9-deaza-8-azaadenosine), 2′-deoxyadenosine, 3′-deoxyadenosine, and 3′-deoxy-3′-aminoadenosine 5′-phosphate, respectively, instead of AMP; tRNA-C-C-A_{oxi} and tRNA-C-C-$A_{oxi\text{-}red}$, tRNA with the 3′-terminal *cis*-diol group oxidized by periodate to a dialdehyde moiety and subsequently reduced by borohydride to two CH_2OH groups.

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nally drawn from Michaelis-Menten kinetic studies of ATP/PP exchange with free enzyme and (E^{Phe}·$tRNA^{Phe}$) complexes (von der Haar & Gaertner, 1975).

In order to determine whether these results are unique to the phenylalanyl-tRNA synthetase:$tRNA^{Phe}$ interaction or whether it is a general phenomenon for all aminoacyl-tRNA synthetases, we have extended our studies to the seryl-, threonyl-, valyl-, and isoleucyl-tRNA synthetases from baker's yeast.

Experimental Section

Materials. $tRNA^{Ser}$-C-C and $tRNA^{Phe}$-C-C were purified from a mixture of tRNAs from baker's yeast (Boehringer, Mannheim, West Germany) as described (Schneider et al., 1972). $tRNA^{Val}$-C-C, $tRNA^{Ile}$-C-C, and $tRNA^{Thr}$-C-C were prepared using basically the same procedure as described (Schneider et al., 1972), namely, BD cellulose chromatography, single step liquid partition and Sephadex A-25 chromatography. This method afforded a preparation of $tRNA^{Val}$ which consisted of both isoacceptors, $tRNA^{Val}_{I}$ and $tRNA^{Val}_{II}$ containing in different batches between 5 and 15% of a $tRNA^{Ile}$. The $tRNA^{Ile}$ fraction obtained contained several—probably three or four—isoacceptors as well as 3–5% of $tRNA^{Val}_{II}$. Since $tRNA^{Val}$ was required free of $tRNA^{Ile}$, and $tRNA^{Ile}$ was needed completely free of $tRNA^{Val}$, the system of salting-out chromatography on Sepharose 4B (Holmes et al., 1975) was adopted. This was performed at room temperature and probably for this reason the ammonium sulfate concentration had to be increased to 1.7 M as compared to 1.3 M (Holmes et al., 1975) in order to bind the tRNA to the column. The buffer employed consisted of 0.01 M sodium acetate (pH 4.5) containing 10^{-2} M magnesium acetate. In this system the constituent $tRNA^{Val}$ and $tRNA^{Ile}$ were eluted as well resolved peaks upon running a gradient decreasing from 1.7 to 1.2 M ammonium sulfate concentration. The order of elution was as follows: peak I, $tRNA^{Val}_{I}$ (main isoacceptor); peak II, $tRNA^{Val}_{II}$ cochromatographing with $tRNA^{Ile}_{I}$ (both minor isoacceptors); peak III, $tRNA^{Ile}_{II}$ (main isoacceptor); and peaks IV and V, $tRNA^{Ile}_{III}$ and $tRNA^{Ile}_{IV}$, respectively (both very minor isoacceptors). In a typical run 5000 A_{260} units of $tRNA^{Val}$ or $tRNA^{Ile}$ fractions were applied to a 2.8 × 50 cm Sepharose 4B column equilibrated with 1.7 M ammonium sulfate in buffer. The column was developed with a gradient from 1.7 to 1.2 M ammonium sulfate in buffer (1.5 L each). The experiments described were performed with $tRNA^{Val}_{I}$ and $tRNA^{Ile}_{II}$, the main isoacceptors. The $tRNA^{Thr}$-C-C was free of any other tRNA; the nature of the particular isoacceptor was not further characterized.

ATP as well as 2′-dATP, FTP, and 3′-deoxy-3′-amino-ATP were incorporated into the different tRNA-C-C as described (Sternbach et al., 1971; Cramer et al., 1975; Maelicke et al., 1974; Fraser & Rich, 1973).

Oxidation and reduction of the 3′-terminal adenosine or formycin respectively was performed according to published procedures (Maelicke et al., 1974; Cramer et al., 1968).

Seryl- (EC 6.1.1.11), valyl- (EC 6.1.1.9), and isoleucyl-tRNA synthetase (EC 6.1.1.5) were prepared as published (von der Haar, 1973). Threonyl-tRNA synthetase (EC 6.1.1.3) cochromatographs with valyl-tRNA synthetase in the salting out system described (von der Haar, 1976b). From this fraction it can be isolated by a single DEAE-cellulose chromatography step. It consists of two identical subunits with a molecular weight of 85 000 ± 5000 for each subunit (unpublished data).

^{14}C-labeled amino acids of Stanstar Grade were purchased from Schwarz Bioresearch, Orangeburg, N.Y.

All other salts and reagents were of the highest purity commercially available.

Methods. (a) Aminoacylation of $tRNA^{Ser}$, $tRNA^{Val}$, and $tRNA^{Ile}$ was performed in 100 μL volume containing 150 mM Tris-HCl buffer (pH 7.6), 150 mM KCl, 10 mM $MgSO_4$, 1.5 mM ATP, and 0.06 mM ^{14}C-labeled amino acid. tRNA and enzyme concentration are specified in the results; 10-μL aliquots were withdrawn and prepared for liquid scintillation counting as described (Schlimme et al., 1968).

(b) Aminoacylation of $tRNA^{Thr}$ was performed as for section a, except that 150 mM Tris-HCl buffer with pH 8.5 instead of 7.5 and 50 mM KCl instead of 150 mM KCl were used.

(c) Aminoacylation under mischarging conditions was performed in 100 μL volume containing 10 mM Tris-HCl buffer (pH 9.0), 8 mM $MgSO_4$, 0.5 mM ATP, and 0.06 mM amino acids. Since the amino acid is added in 10 mM HCl, the actual pH during the test was 8.5. Liquid scintillation counting was performed as for section a.

(d) Data for double-reciprocal plots have been obtained from the linear initial velocities.

(e) Gel Chromatography. A 65 × 1.8 cm Sephadex G-200 column was equilibrated with 0.1 M Tris-HCl buffer (pH 7.6) containing 0.15 M KCl and 0.01 M $MgSO_4$. Phenylalanyl- (0.5 mg) and 0.5 mg of isoleucyl-tRNA synthetase were dissolved together in 0.5 mL of equilibrating buffer and applied to the column. The column was further rinsed with the equilibrating buffer. Fractions (1.9 mL) were collected and 3-μL aliquots of each fraction were assayed for phenylalanyl- and isoleucyl-tRNA synthetase, respectively. The experiments were rerun twice with the equilibrating buffer containing either 7.5 μM $tRNA^{Ile}$-C-C-A or 7.5 μM $tRNA^{Ile}$-C-C-N together with 1 mM ATP and 20 μM isoleucine, respectively.

Results

Throughout this investigation the methodology of steady-state kinetics has been used. Information about the tRNA:enzyme interaction is derived from the "qualitative" differences between the different 3′-end modified substrates or inhibitors rather than from "quantitative" differences. In this sense the results differ from those usually obtained in steady-state kinetic investigations.

(a) *Seryl-tRNA Synthetase.* The K_m for $tRNA^{Ser}$-C-C-A is 0.9 μM. $tRNA^{Ser}$-C-C-3′dA and $tRNA^{Ser}$-C-C-$F_{oxi\text{-}red}$, both of which cannot be aminoacylated, do not inhibit if present at about the same concentration as the substrate. This is contrary to the results obtained with phenylalanyl-tRNA synthetase (von der Haar & Gaertner, 1975). The same is true for $tRNA^{Ser}$-C-C and $tRNA^{Ser}$-C-C-A_{oxi}. Even if these modified $tRNA^{Ser}$-C-C-Ns are present in up to 30-fold excess of the $tRNA^{Ser}$-C-C-A, aminoacylation velocity decreases only very slightly.

Since seryl-tRNA synthetase transfers the serine to the 3′OH of $tRNA^{Ser}$-C-C-A, $tRNA^{Ser}$-C-C-2′dA is a substrate (Cramer et al., 1975) with a K_m of 3 μM, slightly higher than the K_m for $tRNA^{Ser}$-C-C-A. $tRNA^{Ser}$-C-C-F is also a substrate (K_m ≈ 1 μM). $tRNA^{Ser}$-C-C-$A_{oxi\text{-}red}$ is a very weak substrate showing a preparation dependent degree of serylation. For this reason no K_m was determined.

(b) *Threonyl-tRNA Synthetase.* Like seryl-tRNA synthetase (Cramer et al., 1975) threonyl-tRNA synthetase transfers the amino acid to the 3′OH (unpublished). The K_m for $tRNA^{Thr}$-C-C-A and $tRNA^{Thr}$-C-C-2′dA was determined to be 2.5 μM. $tRNA^{Thr}$-C-C and $tRNA^{Thr}$-C-C-3′dA were tested for inhibition in this system. As for seryl-tRNA synthetase a very slight reduction of initial velocity was observed

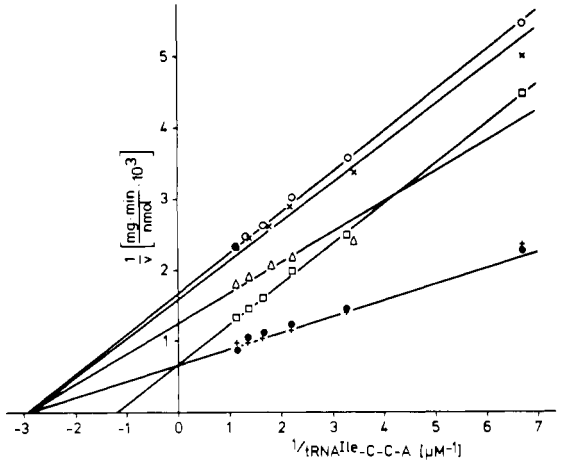


FIGURE 1: Double-reciprocal plot of isoleucylation of $tRNA^{Ile}$-C-C-A in absence and presence of various $tRNA^{Ile}$-C-C-N. Data were obtained from the initial velocity of the aminoacylation assay. (●—●) In absence of inhibitor; (+ — +) in presence of 19 μM $tRNA^{Ile}$-C-C; (□—□) in presence of 3.7 μM $tRNA^{Ile}$-C-C-2′dA; (○—○) in presence of 4.0 μM $tRNA^{Ile}$-C-C-A_{oxi}; (X — X) in presence of 2.3 μM $tRNA^{Ile}$-C-C-$A_{oxi\text{-}red}$; (△ — △) in presence of 0.95 μM $tRNA^{Ile}$-C-C-$F_{oxi\text{-}red}$. Enzyme concentration was 0.24 μg per 100-μL assay.

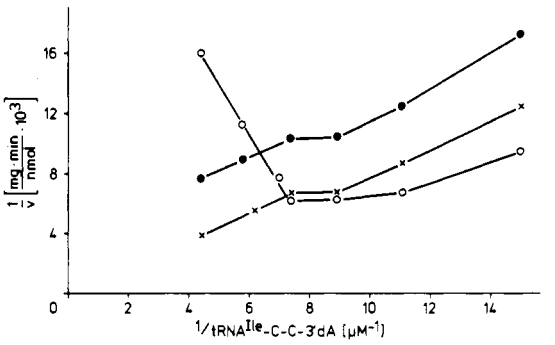


FIGURE 2: Double-reciprocal plot of isoleucylation of $tRNA^{Ile}$-C-C-3′dA (○—○) in absence and presence of $tRNA^{Ile}$-C-C (+ — +) and $tRNA^{Ile}$-C-C-2′dA (●—●) respectively. Data were taken from the initial velocities of the aminoacylation assay. Concentration of $tRNA^{Ile}$-C-C was 3.7 μM and of $tRNA^{Ile}$-C-C-2′dA, 4.6 μM. Enzyme concentration was 0.15 μg per 100-μL assay.

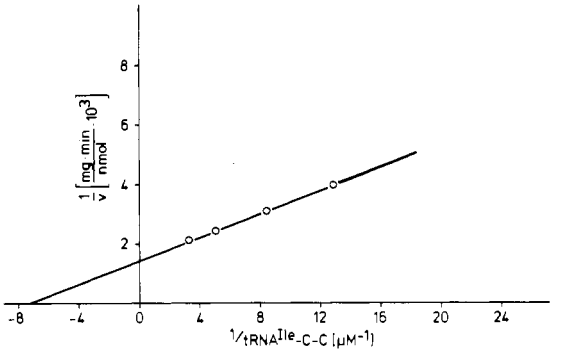


FIGURE 3: Double-reciprocal plot of isoleucylation of $tRNA^{Ile}$-C-C-3′dA at constant concentration in presence of increasing amounts of $tRNA^{Ile}$-C-C. Data were taken from the initial velocities of the aminoacylation assay. Concentration of $tRNA^{Ile}$-C-C-3′dA was 5.8 μM, of enzyme 0.18 μg per 100-μL assay.

only if they were present in a 20-fold excess over $tRNA^{Thr}$-C-C-A.

(c) *Valyl-tRNA Synthetase.* Valyl-tRNA synthetase transfers the valine to the 2′OH as does phenylalanyl-tRNA synthetase (Cramer et al., 1975). Nevertheless, with respect to inhibition by 3′-terminal modified tRNAs, it behaves identically with the 3′OH acceptors $tRNA^{Ser}$ and $tRNA^{Thr}$. The K_m for the substrates $tRNA^{Val}$-C-C-A, $tRNA^{Val}$-C-C-3′dA, and $tRNA^{Val}$-C-C-F are identical within the limits of error (3 ± 1 μM). All inhibitors tested—$tRNA^{Val}$-C-C, $tRNA^{Val}$-C-C-2′dA, $tRNA^{Val}$-C-C-$A_{oxi\text{-}red}$, $tRNA^{Val}$-C-C-$F_{oxi\text{-}red}$—showed only a very small reduction in the initial velocity of aminoacylation if present in high excess (20–30-fold) compared with the substrates.

(d) *Isoleucyl-tRNA Synthetase.* Isoleucyl-tRNA synthetase, which transfers isoleucine to the 2′OH of $tRNA^{Ile}$-C-C-A (Cramer et al., 1975), shows completely different behavior with respect to the response on modification of the 3′-terminal adenosine when compared with seryl-, valyl-, and threonyl-tRNA synthetases. Although in many respects it resembles phenylalanyl-tRNA synthetase it still shows some differences (von der Haar & Gaertner, 1975). $tRNA^{Ile}$-C-C-A (Figure 1) and $tRNA^{Ile}$-C-C-F both have a K_m of 3 ∓ 1 μM. Isoleucylation of $tRNA^{Ile}$-C-C-A is only slightly inhibited by a high concentration of $tRNA^{Ile}$-C-C (Figure 1). As in the phenylalanyl system $tRNA^{Ile}$-C-C-2′dA lacking the accepting 2′OH of the 3′ terminus is a competitive inhibitor with K_i = 2 μM. Contrary to the phenylalanyl system $tRNA^{Ile}$-C-C-A_{oxi} and $tRNA^{Ile}$-C-C-$F_{oxi\text{-}red}$ are classical noncompetitive inhibitors with K_i = 2 ± 1 μM. The data for $tRNA^{Ile}$-C-C-$A_{oxi\text{-}red}$ do not allow us to decide whether it is a noncompetitive or uncompetitive inhibitor. Nevertheless $tRNA^{Ile}$-C-C-$A_{oxi\text{-}red}$ inhibits to the same extent as do the noncompetitive inhibitors (Figure 1).

In contrast to $tRNA^{Ile}$-C-C-A, $tRNA^{Ile}$-C-C-3′dA shows substantial substrate inhibition at moderate concentration (Figure 2). Isoleucylation of $tRNA^{Ile}$-C-C-3′dA is only slightly inhibited by $tRNA^{Ile}$-C-C and is inhibited uncompetitive by $tRNA^{Ile}$-C-C-2′dA. Because of the substrate inhibition K_m and K_i were not determined. A very striking observation is (Figure 2) that substrate inhibition of $tRNA^{Ile}$-C-C-3′dA is suppressed by the presence of $tRNA^{Ile}$-C-C as well as $tRNA^{Ile}$-C-C-2′dA.

Since $tRNA^{Ile}$-C-C suppresses the substrate inhibition of $tRNA^{Ile}$-C-C-3′dA, this phenomenon enables one to determine the interaction of $tRNA^{Ile}$-C-C with isoleucyl-tRNA synthetase in comparison with $tRNA^{Ile}$-C-C-3′dA (Figure 3). Employing a high constant concentration of $tRNA^{Ile}$-C-C-3′dA the suppression of substrate inhibition caused by increasing amount of $tRNA^{Ile}$-C-C can be analyzed using the usual double-reciprocal plot (Figure 3). Thus an interaction constant of 1.5 μM for $tRNA^{Ile}$-C-C is obtained. Hence, despite the fact that $tRNA^{Ile}$-C-C does not inhibit isoleucylation it interacts with the enzyme as efficiently as the substrate.

Transfer of valine misactivated by isoleucyl-tRNA synthetase to $tRNA^{Ile}$-C-C-3′dA (von der Haar & Cramer, 1975) can serve as a further tool to investigate interaction of $tRNA^{Ile}$-C-C-3′dA with isoleucyl-tRNA synthetase. The K_m for valylation of $tRNA^{Ile}$-C-C-3′dA is within the limits of error the same as for isoleucylation of $tRNA^{Ile}$-C-C-A. Substrate inhibition by $tRNA^{Ile}$-C-C-3′dA is also observed in this reaction (Figure 4). $tRNA^{Ile}$-C-C-A from which valine is hydrolyzed prior to release of Val-$tRNA^{Ile}$-C-C-A (von der Haar & Cramer, 1976) inhibits the reaction in a noncompetitive manner. In this case K_i for $tRNA^{Ile}$-C-C-A is 4 μM (Figure 4).

The results obtained with different systems are summarized in Table I.

(e) *Molecular Weight of Isoleucyl-tRNA Synthetase.* Free

TABLE I: Behavior of tRNA-C-C-N on Interaction with Cognate Aminoacyl-tRNA Synthetase.

enzyme	tRNA-C-C-A	tRNA-C-C	tRNA-C-C-F	tRNA-C-C-2'dA	tRNA-C-C-3'dA	tRNA-C-C-A_{oxi}	tRNA-C-C-$A_{oxi-red}$	tRNA-C-C-$F_{oxi-red}$
Ser-tRNA synthetase	substrate K_m = 0.9 μM	weak[a] inhibitor	substrate K_m = 1 μM	substrate K_m = 3 μM	weak inhibitor	weak inhibitor	substrate K_m, ND	weak inhibitor
Thr-tRNA synthetase	substrate K_m = 2.5 μM	weak inhibitor	ND[c]	substrate K_m = 2.5 μM	weak inhibitor	ND	ND	ND
Val-tRNA synthetase	substrate K_m = 3 μM	weak inhibitor	substrate K_m = 3 μM	weak inhibitor	substrate K_m = 3 μM	weak inhibitor	weak inhibitor	weak inhibitor
Ile-tRNA synthetase	substrate K_m = 3 μM	weak inhibitor	substrate K_m = 3 μM	competitive inhibitor K_i = 2 μM	substrate K_m, ND	noncompetitive inhibitor K_i = 2 μM	noncompetitive inhibitor K_i = 2 μM	noncompetitive inhibitor K_i = 2 μM
Phe-tRNA synthetase	substrate[b] K_m = 2.5 μM	weak inhibitor	substrate K_m = 2.5 μM	competitive inhibitor K_i = 0.28 μM	substrate K_m = 2.5 μM	mixed type[b] inhibitor	substrate K_m = 2.5 μM	competitive inhibitor K_i = 0.45 μM

[a] For the definition of a weak inhibitor, see Results. [b] For details, see von der Haar & Gaertner (1975). [c] ND, not determined.

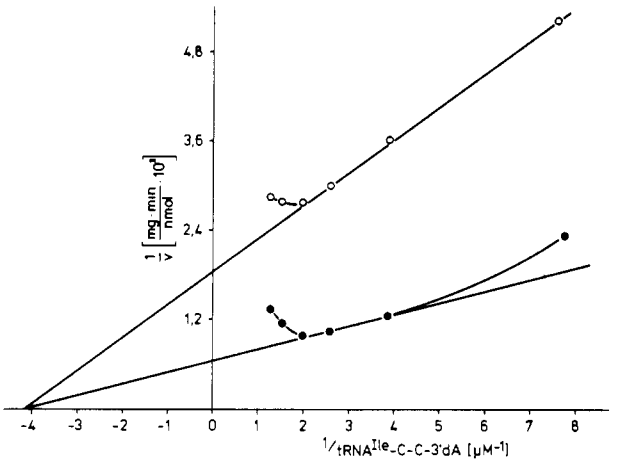


FIGURE 4: Double-reciprocal plot of valylation of $tRNA^{Ile}$-C-C-3'dA in absence (●—●) and presence (○—○) of $tRNA^{Ile}$-C-C-A. Data were taken from the initial velocities of the aminoacylation assay. Concentration of $tRNA^{Ile}$-C-C-A was 8.4 μM, of valine 60 μM, and of enzyme 9.2 μg per 100-μL assay.

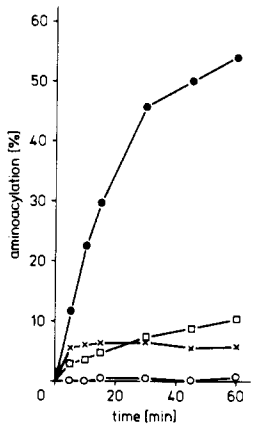


FIGURE 5: Misaminoacylation of $tRNA^{Phe}$-C-C-N by isoleucyl-tRNA synthetase. Enzyme concentration was 13 μg per 100-μL assay in all cases. Reaction was performed under mischarging conditions (Methods). One hundred percent aminoacylation was assumed to be 1.65 nmol/A_{260} unit. (○—○) $tRNA^{Phe}$-C-C-F; (X—X) $tRNA^{Phe}$-C-C-A; (□—□) $tRNA^{Phe}$-C-C-3'NH_2A; (●—●) $tRNA^{Phe}$-C-C-3'dA.

isoleucyl-tRNA synthetase from baker's yeast exists as a single chain enzyme with molecular weight 115 000 (von der Haar & Cramer, 1976). To answer the question as to whether it dimerizes as does lysyl-tRNA synthetase (Österberg et al., 1975) on complexation with tRNA the experiment described below was undertaken. Phenylalanyl-tRNA synthetase with a molecular weight of 260 000 was cochromatographed on a Sephadex G-200 column with isoleucyl-tRNA synthetase. Both activities are well separated during this procedure. There is no difference in the elution volume of either of the enzymes on addition of $tRNA^{Ile}$-C-C-A or $tRNA^{Ile}$-C-C-A plus ATP plus isoleucine, indicating that the binary as well as the ternary complex does not undergo dimerization.

(f) *Misaminoacylation of $tRNA^{Phe}$-C-C-N by Isoleucyl-tRNA Synthetase.* Valyl- and phenylalanyl-tRNA synthetases both from baker's yeast (Ebel et al., 1973; von der Haar, 1976c), misaminoacylate noncognate $tRNA^{Ile}$-C-C-A. However, isoleucyl-tRNA synthetase from baker's yeast is unable to misaminoacylate $tRNA^{Phe}$-C-C-A (Figure 5). The same is true for $tRNA^{Phe}$-C-C-F, $tRNA^{Phe}$-C-C-$A_{oxi-red}$, and $tRNA^{Phe}$-C-C-3'NH_2A. Because the tRNAs containing an aminoadenosine at the 3' terminus are not accessible to hy-

drolytic correction (von der Haar & Cramer, 1976), this result cannot be due to hydrolytic correction. In contrast, $tRNA^{Phe}$-C-C-3′dA is readily isoleucylated up to a level of 56% under the conditions of our experiment (Figure 5). It should be noted that contrary to other investigators (Ebel et al., 1973) we avoided the presence of organic solvents in the assay. The misaminoacylation was achieved by keeping the ionic strength in the assay at a low level. For comparison it should be noted that with valyl-tRNA synthetase the rate of misaminoacylation of $tRNA^{Ile}$-C-C-A is at least tenfold faster than the rate of valylation of $tRNA^{Ile}$-C-C-3′dA (unpublished).

Discussion

(a) *The Experimental Approach.* Earlier investigations had shown that even single chain enzymes possess more than one binding site for the small substrates amino acid and ATP (Fersht, 1975) and that the binding sites interact in a complex manner (Fersht, 1975; Mulvey & Fersht, 1977). Additionally, in the phenylalanyl system from baker's yeast (von der Haar & Gaertner, 1975; Krauss et al., 1976) as well as in the isoleucyl system from *E. coli* (Yarus & Berg, 1969) there is an influence between tRNA and the small substrates, indicating that reactivity and specificity are strongly interrelated (von der Haar & Gaertner, 1975). Therefore we have investigated the influence of modification at the 3′-terminal adenosine (the reacting moiety) of tRNA with aminoacyl-tRNA synthetase in the complete synthesizing system rather than simply studying only binary complex formation between tRNA and free enzyme, the approach which has been mainly used (von der Haar, 1976d; Riegler et al., 1976; Krauss et al., 1976; 1977). The only way to achieve this at the moment is a Michaelis–Menten type investigation in which information can be obtained from the qualitative differences exhibited during interaction of 3′-terminal modified tRNA-C-C-Ns with the enzyme (von der Haar & Gaertner, 1975).

(b) *Reacting Site Triggering by the 3′-Terminal Adenosine with Seryl-, Threonyl-, and Valyl-tRNA Synthetase.* In contrast to the phenylalanyl system, in which $tRNA^{Phe}$-C-C-2′dA lacking the accepting 2′OH was a strong competitive inhibitor, the analogous $tRNA^{Val}$-C-C-2′dA in the valyl system and the $tRNA^{Ser}$-C-C-3′dA and $tRNA^{Thr}$-C-C-3′dA in the seryl and threonyl system, respectively, showed only very weak inhibition. Similar weak inhibition was observed with all other tRNA-C-C-Ns tested in these systems.

In the phenylalanyl system we were able to show by a detailed analysis (von der Haar & Gaertner, 1975) that a primarily formed catalytically incompetent [$tRNA^{Phe}$-C-C-A·E^{Phe}] complex was triggered to a catalytically competent complex by the 3′-terminal adenosine, a fact later confirmed by other investigators (Krauss et al., 1976, 1977). The data indicated the particular importance of the ribose as compared with the base portion of the 3′-adenosine (von der Haar & Gaertner, 1975). In analogy to the observations in the phenylalanyl system one would also expect inhibition by the respective tRNA-C-C-N lacking the accepting OH in the systems investigated here. Since this is not the case, one possible explanation is that these modified tRNA-C-C-Ns do not bind to the respective enzymes. This possibility can be ruled out since they are as efficient as the tRNA-C-C-A in eluting aminoacyl-tRNA synthetases from cation exchangers (affinity elution) (von der Haar, 1976d; von der Haar, 1973). Furthermore these modified tRNA-C-C-N are very effective in the inhibition of misaminoacylation of noncognate tRNA-C-C-A (unpublished). The alternative which remains is that in the seryl, threonyl, and valyl system a triggering by the 3′-terminal adenosine exists and that it is intimately related to the presence of the accepting hydroxyl. Since in this system the triggering and accepting functions are related to the same group a further investigation with the methodology chosen here is impossible.

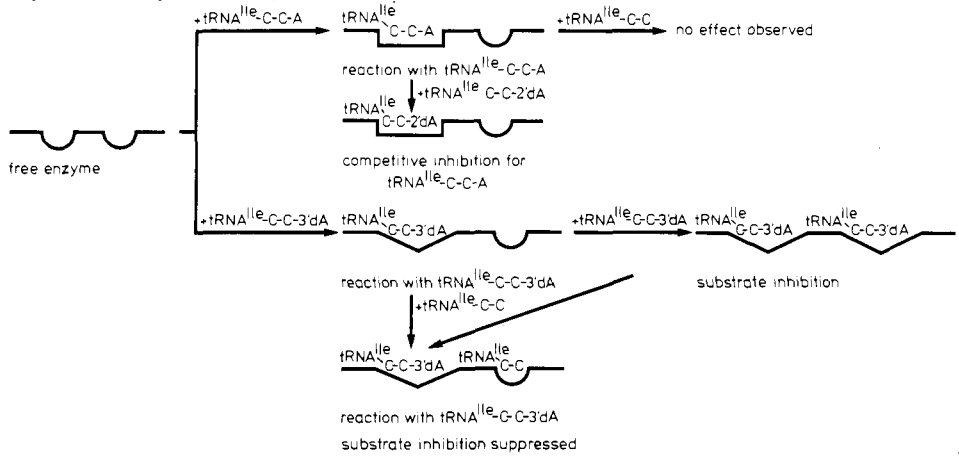
(c) *Isoleucyl-tRNA Synthetase.* The results presented for isoleucyl-tRNA synthetase allow the following conclusions to be drawn.

(1) The enzyme has two binding sites for tRNA per single chain of molecular weight 115 000.

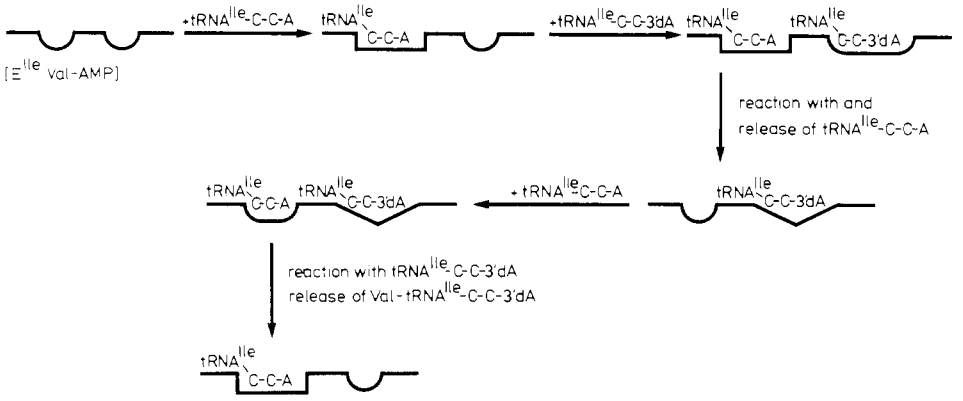
The purely noncompetitive inhibition by $tRNA^{Ile}$-C-C-A_{oxi}, $tRNA^{Ile}$-C-C-$A_{oxi\text{-}red}$, and $tRNA^{Ile}$-C-C-$F_{oxi\text{-}red}$ is most easily explained by assuming a second binding site on the enzyme besides the site at which $tRNA^{Ile}$-C-C-A is aminoacylated (Figure 1). The same is true for the uncompetitive inhibition of $tRNA^{Ile}$-C-C-3′dA by $tRNA^{Ile}$-C-C-2′dA (Figure 2). An experimental fact, which undoubtedly points to the existence of two binding sites for $tRNA^{Ile}$ per enzyme molecule, is the substrate inhibition exhibited by $tRNA^{Ile}$-C-C-3′dA as well as the suppression of this substrate inhibition by $tRNA^{Ile}$-C-C (Figure 2). Phenotypically in this case $tRNA^{Ile}$-C-C acts like an effector for isoleucylation of $tRNA^{Ile}$-C-C-3′dA and the only way in which an effector can work is to occupy a site on the enzyme other than the reactive site.

In light of the fact that for isoleucyl-tRNA synthetase from *E. coli* and *B. stearothermophilus* only a single binding site for tRNA (Yarus & Berg, 1967, 1969; Charlier & Grosjean, 1972) and isoleucine (Berthelot & Yaniv, 1970; Rainey et al., 1976) per 110 000 molecular weight has been determined and since lysyl-tRNA synthetase tends to dimerize on complexation with $tRNA^{Lys}$ (Österberg et al., 1975) the question arises as

SCHEME I: Reactivity of Isoleucyl-tRNA Synthetase with Different $tRNA^{Ile}$-C-C-N.



SCHEME II: Valylation of tRNA ^{Ile}-C-C-3′dA in the Presence of the tRNA^{Ile}-C-C-A.



to whether the isoleucyl-tRNA synthetase also dimerizes on complexation with tRNA^{Ile}. This possibility can be excluded from the gel chromatographic behavior described above. Even in the presence of all substrates in which a synthesizing system is known to be chromatographed (von der Haar & Cramer, 1976), no evidence for dimerization is seen. Hence isoleucyl-tRNA synthetase has two binding sites for tRNA^{Ile} per single chain of molecular weight 115 000.

(2) Binding of tRNA to and reactivity in each binding site are related to conformational changes induced by the nonaccepting 3′OH of the 3′-terminal adenosine.

In a purely formal way the interplay of tRNA^{Ile}-C-C-N in the two binding sites is depicted in Scheme I. The free enzyme possesses two binding sites, which are identical but catalytically inactive. After binding of one tRNA^{Ile}-C-C-A or of the competitive inhibitor tRNA^{Ile}-C-C-2′dA (the tRNA lacking the accepting 2′OH but possessing the nonaccepting 3′OH) to one of the binding sites this site is transformed to a catalytically active form. Simultaneously with this transformation the second binding site is influenced in such a way that interaction with the second tRNA^{Ile} can no longer be observed. This could explain the fact that only one binding site has been observed in all investigations so far performed (Yarus & Berg, 1967, 1969; Charlier & Grosjean, 1972; Hustedt & Kula, 1977) with tRNA^{Ile}-C-C-A and the enzyme from *E. coli* and *B. stearothermophilus*. However, one has to be cautious, since comparison of identical enzymes from different organisms may be erroneous. The transformation of the binding site from a catalytically inactive to an active form must be due to a triggering by the nonaccepting 3′OH of the 3′-terminal ribose, since the behavior described above is observed only with those tRNA^{Ile}-C-C-N, which possess this particular 3′OH.

If a tRNA lacking the 3′OH is bound, as is the case for the substrate tRNA^{Ile}-C-C-3′dA, the formerly inactive binding site of the enzyme is triggered to a form which is catalytically active, but with this substrate the second binding site remains open. Hence a second tRNA^{Ile}-C-C-3′dA is bound, which also triggers the second site (Scheme I). As a consequence, substrate inhibition is observed. The suggestion that the conformation of the catalytic site occupied by tRNA^{Ile}-C-C-3′dA is different from the conformation of the site occupied by tRNA^{Ile}-C-C-A (Scheme I) is supported by two observations. Firstly, substrate inhibition can be suppressed by complexation with a tRNA^{Ile}-C-C. This species is obviously unable to perform the transformation from the inactive to the catalytically active state. Being, however, bound to the enzyme, it can compete preferentially with tRNA^{Ile}-C-C-3′dA (Figure 3 and Scheme I). Secondly, misisoleucylation is observed only with the tRNA^{Phe}-C-C-3′dA and not with tRNA^{Phe}-C-C-A (see below).

(3) Isoleucyl transfer to tRNA^{Ile}-C-C-A can be performed only at one site at a time.

Isoleucyl-tRNA synthetase from different sources is known to misactivate valine (von der Haar & Cramer, 1976) and this misactivated valine can be transferred to tRNA^{Ile}-C-C-3′dA and tRNA^{Ile}-C-C-A. However, in the latter case a hydrolysis of the Val-tRNA^{Ile}-C-C-A occurs regenerating tRNA^{Ile}-C-C-A (von der Haar & Cramer, 1975, 1976). For an enzyme with a single binding site or with two binding sites acting independently, tRNA^{Ile}-C-C-A should be a competitive inhibitor for the valylation of tRNA^{Ile}-C-C-3′dA. Experimentally it is observed, however (Figure 4), that the tRNA^{Ile}-C-C-A causes noncompetitive inhibition of the valylation of tRNA^{Ile}-C-C-3′dA. Hence both sites on the enzyme cannot act independently. The noncompetitive inhibition is most easily explained by assuming that, as shown in Scheme II, transfer of the amino acid can be performed only at one binding site at the time. This is in agreement with the behavior of valyl-tRNA synthetase from *E. coli* toward the small substrates ATP and amino acid. For this system it was shown that one valyl adenylate can be formed, whereas at the same time a second valine as well as ATP can be bound per single chain enzyme forming a valyl adenylate only very slowly (Fersht et al., 1975; Mulvey & Fersth, 1977).

(4) The conformational change induced by the nonaccepting 3′OH of the 3-terminal adenosine is a prerequisite for specific isoleucylation of tRNA^{Ile}-C-C-A.

tRNA^{Phe}-C-C-A as well as tRNA^{Phe}-C-C-3′NH_2A are only misisoleucylated by isoleucyl-tRNA synthetase to a negligible extent, whereas tRNA^{Phe}-C-C-3′dA lacking the nonaccepting triggering 3′OH is readily misaminoacylated by isoleucyl-tRNA synthetase (Figure 5). Hence the triggering by the 3′OH seems to play a dual role; with the noncognate tRNA^{Phe} it prevents misisoleucylation, with the cognate tRNA^{Ile} it enhances the specificity of isoleucylation of tRNA. In this context it is of interest that valyl-tRNA synthetase, which is triggered by the accepting 2′OH rather than by the nonaccepting OH (see above), is more effective in misaminoacylating tRNA^{Phe}-C-C-A than tRNA^{Phe}-C-C-3′dA (unpublished), pointing again to the exceptional role played by the nonaccepting OH in the isoleucyl system.

We had observed earlier that the nonaccepting 3′OH is essential for the correction of misactivation of valine by isoleucyl-tRNA synthetase (von der Haar & Cramer, 1976). Are these findings related to the triggering by the nonaccepting 3′OH described in the present paper? As far as we see the results presented here are in agreement with the interpretations published earlier. The "switching on" of catalytic activity caused by the nonaccepting OH is a special phenomenon for isoleucyl-tRNA synthetase. The chemical proofreading (von

der Haar & Cramer, 1976) leading to correction of misactivation of an amino acid is observed, however, for at least two other synthetases, the valyl-tRNA synthetase from baker's yeast (Igloi et al., 1977) as well as from *E. coli* (Fersht & Kaethner, 1976) and for phenylalanyl-tRNA synthetase from baker's yeast (G. L. Igloi, F. von der Haar, & F. Cramer, in press). Hence we conclude that the reacting site triggering described above enhances the specificity of aminoacylation of a particular tRNA, whereas the chemical proofreading mechanism (von der Haar & Cramer, 1976) enhances the specificity with respect to the amino acid. A possible interrelation of both events, however, cannot be finally excluded.

In summary, we would like to state that triggering of an aminoacyl-tRNA synthetase by the invariant 3′-terminal adenosine—the reactive residue of the tRNA—has been observed in five cases. With phenylalanyl-tRNA synthetase the total ribose moiety seems to be involved (von der Haar & Gaertner, 1975). With seryl-, threonyl-, and valyl-tRNA synthetase the accepting hydroxyl of the 3′-terminal ribose must serve as a switch for catalytic activity, whereas with isoleucyl-tRNA synthetase besides the total 3′-terminal adenosine the nonaccepting hydroxyl is in particular responsible for this control step.

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