Biochemistry

 Volume 20, Number 8 April 14, 1981

# Catalytic Mechanism of Valyl-tRNA Synthetase from Baker's Yeast. Reaction Pathway and Rate-Determining Step in the Aminoacylation of $tRNA^{Val}$†

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ABSTRACT: The catalytic mechanism of valyl-tRNA synthetase from baker's yeast has been investigated by pre-steady-state and steady-state kinetic measurements and end product dissociation studies. The pre-steady-state kinetics show a lag period during the early time when the reaction is started with free enzyme. The preincubation of the synthetase with $tRNA^{Val}$ and/or valine or preformation of Val~AMP leads to a progressive suppression of the lag. This lag probably reflects conformational transitions of the enzyme·substrate complex necessary for the transfer. At low pH or at low ionic strength, the $tRNA^{Val}$ charging occurs much faster at the pre steady state than at the steady state. We show that after the fast transfer of valine from adenylate to $tRNA^{Val}$, followed by the fast dissociation of AMP and $PP_i$, a new adenylate is synthesized which promotes the dissociation of the nascent Val-$tRNA^{Val}$. This dissociation occurs in a multistep process. First ATP and magnesium promote the ejection of the valine moiety of Val-$tRNA^{Val}$ from the adenylate site. A new adenylate is then synthesized which promotes, in the presence of magnesium, several state changes of the end product complex. A complex is finally generated in which the enzyme-bound Val-$tRNA^{Val}$ is able to exchange rapidly with a $tRNA^{Val}$ molecule. The free $tRNA^{Val}$ plays an active role in this exchange. Depending upon the experimental conditions, one of these steps can determine the steady-state rate of $tRNA^{Val}$ charging. The dissociations of enzyme-bound uncharged $tRNA^{Val}$ or aa-tRNAs substituted on the amino acid or on the tRNA parts by noncognate parts as well as the effect of the replacement of the adenylate by wrong adenylates have been investigated. It is shown that the valine and the tRNA moieties of Val-$tRNA^{Val}$ and the valine moiety of the adenylate are involved in this mechanism of dissociation. Finally, the rate-determining step of the reversal of $tRNA^{Val}$ charging at the steady-state has been investigated. It is shown that this step is the dissociation of the deacylated $tRNA^{Val}$ from enzyme.

The mechanism of aminoacylation of tRNAs[1] by aminoacyl-tRNA synthetases has up to today been widely investigated. For practical reasons, these investigations have largely been done by steady-state kinetic measurements [see the reviews by Söll & Schimmel (1974), Kisselev & Favorova (1974), Kalousek & Konigsberg (1975), and Schimmel & Söll (1979)]. They aimed to resolve some mechanistic aspects for several synthetases, such as the order of addition of the substrates and release of the end products as well as the nature of the pathway used in the overall tRNA charging [i.e., Allende et al. (1970) and Papas & Mehler (1971)]. However, there are limitations to these approaches (Cleland, 1970; Merault et al., 1978). Discrete and transient steps involving either enzyme state changes or highly labile intermediates, which are not related to associations of substrates or releases of end products, cannot be accurately detected in this way. Under adequate circumstances, however, such stages can be detected by pre-steady-state kinetics. For this reason, the introduction of rapid kinetic techniques in the synthetases field, in particular quenched flow, contributed largely to the elucidation of the mechanism of these enzymes and particularly to the establishment of the two-step aminoacylation pathway (Fersht & Kaethner, 1976; Fersht et al., 1978) (eq 1):

$$E + aa + ATP \underset{}{\overset{MgCl_2}{\rightleftharpoons}} E{\cdot}AMP{\sim}aa + PP_i$$

$$E{\cdot}AMP{\sim}aa + tRNA \rightarrow aa\text{-}tRNA + AMP + E \quad (1)$$

One of the kinetic aspects in tRNA charging which cannot be easily investigated by usual kinetic measurements concerns the nature of the rate-determining step at the steady state. This could be, a priori, the association of the substrates to the enzyme, one of the two catalytic steps in eq 1, the dissociation

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[1] Abbreviations used: tRNA, transfer ribonucleic acids; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Val-$tRNA^{Val}$, $tRNA^{Val}$ charged with valine; Val-$tRNA^{Phe}$, $tRNA^{Phe}$ charged with valine; Phe-$tRNA^{Val}$, $tRNA^{Val}$ charged with phenylalanine; Thr, threonine; Ala, alanine; [$^3$H]At$RNA^{Val}$, $tRNA^{Val}$ labeled with [$^3$H]adenosine on its 3′ end.

0006-2960/81/0420-2065$01.25/0 

of one of the end-products, or finally any other stage involved in the overall process. It is true for a number of synthetases that the association of substrates is diffusion-controlled (Holler, 1978) and that the activation of amino acid (the first step in eq 1) occurs significantly faster than the tRNA charging (see the general reviews). For these reasons the attention focuses particularly on the transfer of the activated amino acid from aminoacyladenylate to the tRNA (second step in eq 1) for the rate-determining step investigation. This viewpoint is supported by the reports of Eldred & Schimmel (1972) and Carr et al. (1975), showing that at least under some experimental conditions, the dissociation of the enzyme-bound Ile-tRNA$^{Ile}$ can be rate determining at the steady state in the *Escherichia coli* isoleucylation system. Arguments for a similar behavior in the yeast arginylation system have been reported by Fersht et al. (1978).

Besides this kinetic aspect of synthetases, the search for and the study of possible other stages involved in the overall tRNA charging process are also of a great interest. There is much evidence that conformational changes occur during the catalytic process [see the general review by Schimmel & Söll (1979)]; in some cases they have been firmly established (Von der Haar & Gaertner, 1975; Von der Haar & Cramer, 1978a,b; Wehmeyer et al., 1979).

We report here a pre-steady-state and a steady-state kinetic investigation of the tRNA charging reaction catalyzed by the yeast valyl-tRNA synthetase. A number of interesting questions and ideas are attacked in this study; e.g., what is the elementary step which is rate determining in the tRNA$^{Val}$ charging reaction? Is this step the same at the pre steady state and at the steady state? We designed experiments to determine these steps. In addition to the tRNA charging reaction we also focus our attention on the consumption of ATP and the appearance of adenylate and AMP. It is thus possible to learn whether a new Val~AMP molecule is synthesized on the enzyme prior the release of the enzyme-bound nascent Val-tRNA$^{Val}$ and if so, if Val~AMP promotes this release and by what mechanism. Since the enzyme-bound adenylate and aa-tRNA can be retained on nitrocellulose disks, the steady-state concentration of the various enzyme complexes could be determined. Finally, the comparison of the kinetic properties of various enzyme complexes involved in the catalytic process with those of complexes generated in situ by mixing the free reactants allowed us to test whether these complexes were transconformed during the catalytic process.

## Experimental Procedures

*General.* L-[$^{14}$C]Valine (sp act., 250 mCi/mmol) and [$^{14}$C]ATP (sp act., 200 mCi/mmol) were from the Commissariat à l'Energie Atomique. [$^{32}$P]PP$_i$ was from Amersham, France. [$\alpha$-$^{32}$P]- and [$\gamma$-$^{32}$P]ATP were from New England Nuclear. ATP and inorganic pyrophosphatase (1 unit catalyzes the formation of 1 $\mu$mol of inorganic phosphate/min at 25 °C and pH 7.2) were from Sigma Chemical Co., and unfractionated yeast tRNA was from Boehringer. Sephadex G-75 Superfine was from Pharmacia, silica gel sheets 60F 254 were from Merck, nitrocellulose disks (pore size, 0.45 $\mu$m) were from Millipore, and glass-fiber disks, GF/c, were from Whatman.

*Enzymes and tRNAs.* Valyl-tRNA synthetase (EC 6.1.1.9) from yeast was prepared as described by Kern et al. (1975). The turnover, measured in the presence of the standard aminoacyalation mixture (Kern et al., 1975) at 37 °C and pH 7.2, was of 8.0 s$^{-1}$. The molar enzyme concentrations were determined from the absorbance ($A_{280nm}$ = 1.79 mg mL$^{-1}$ cm$^{-1}$) by using a molecular weight of 130 000.

The enzyme·Val~AMP complexes labeled either on the amino acid or on the AMP moiety were prepared in 50 mM NaHepes, pH 7.2, in the presence of 25 $\mu$M valyl-tRNA synthetase, either 50 $\mu$M valine [$^3$H-labeled (210 cpm/pmol) or $^{14}$C-labeled (27 cpm/pmol)] and 1 mM ATP or 1 mM valine and 50 $\mu$M ATP [$\alpha$-$^{32}$P-labeled (33 cpm/pmol)], 2 mM $MgCl_2$, and 10 units of inorganic pyrophosphatase per mL. The complex was separated from the free substrates by gel filtration on a Sephadex G-75 column.

Purified yeast tRNA$^{Val}$ (acceptance capacity, 1500 pmol/$A_{260nm}$) was obtained as described by Kern et al. (1975); yeast tRNA$^{Phe}$ (acceptance capacity, 1300 pmol/$A_{260nm}$) was obtained pure after countercurrent fractionation (Dirheimer & Ebel, 1967). Unlabeled Val-tRNA$^{Val\,1}$, [$^{14}$C]Val-tRNA$^{Val}$ (272 cpm/pmol), Val-[$^3$H]AtRNA$^{Val\,1}$ (39 cpm/pmol), [$^{14}$C]Val-tRNA$^{Phe\,1}$ (135 cpm/pmol), and [$^{14}$C]Phe-tRNA$^{Val\,1}$ (122 cpm/pmol) were prepared in large scale as described by Kern et al. (1972) and isolated according to Yang & Novelli (1968). [$^3$H]AtRNA$^{Val\,1}$ was prepared as described by Renaud et al. (1979). 5′-$^{32}$P labeling of tRNA$^{Val}$ was performed according to Silberklang et al. (1977). tRNA$^{Val}$ was oxidized in the presence of 2 mM sodium periodate according to the procedure described by Kern et al. (1972).

*Aminoacylation Kinetics.* Rapid kinetic experiments were performed with a quenched-flow apparatus constructed by one of us (J.G.) and whose principle will be described elsewhere. The reaction was started by mixing the contents of two syringes (2 × 500 $\mu$L) and stopped after increasing times (from 6.7 to 400 ms) by quenching with 20% trichloroacetic acid contained in a third syringe. The final reaction mixtures contained, unless otherwise indicated, 50 mM NaHepes,[1] pH 7.2, 10 $\mu$M tRNA$^{Val}$, 100 $\mu$M L-[$^{14}$C]valine (195 cpm/pmol), 5 mM ATP, 20 mM $MgCl_2$, 0.5 mM dithioerythritol, 0.92 $\mu$M valyl-tRNA synthetase, and inorganic pyrophosphatase as indicated. The content of each syringe was preincubated for 5 min at 25 °C to allow the action of the pyrophosphatase, when present, and the kinetics were then conducted at 22 °C. The tRNA and [$^{14}$C]Val-tRNA$^{Val}$ precipitates were filtered through glass fiber disks, GF/c. After being washed 4 times with 5% trichloroacetic acid and with ethanol, the disks were dried and then counted by liquid scintillation. The steady-state kinetics were conducted by hand under similar conditions unless otherwise indicated. The labeled Val-tRNA$^{Val}$ synthesized was determined as described previously (Kern et al., 1975).

*Kinetics of [$\gamma$-$^{32}$P]ATP Cleavage.* The kinetics were conducted by quenched flow at 22 °C. The final reaction mixture contained 50 mM NaHepes, pH 7.2, 4.2 $\mu$M [$\gamma$-$^{32}$P]ATP (13 200 cpm/pmol), 100 $\mu$M L-valine, 20 mM $MgCl_2$, 0.5 mM dithioerythritol, 0.92 $\mu$M valyl-tRNA synthetase, and 10 units of inorganic pyrophosphatase per mL and, when present, 10 $\mu$M tRNA$^{Val}$. After reaction times ranging from 6.7 to 81 ms, the mixture was quenched with 20% trichloroacetic acid. An aliquot of each quenched fraction was then mixed with 400 $\mu$L of a suspension of acid-washed Norit (5% w/v) containing 7% perchloric acid and 0.1 M PP$_i$. After centrifugation, the [$^{32}$P]PP$_i$ was determined by liquid scintillation after dilution of 300 $\mu$L of the supernatant in the Bray's solution. The rate constants of valine activation were determined from rates of ATP cleavage corrected for a saturating concentration of ATP ($K_m$ = 200 and 660 $\mu$M in the absence and in the presence of tRNA$^{Val}$, respectively, as determined by the PP$_i$–ATP exchange; Kern & Giegé, 1979).

*Parallel Measurements of Val-tRNA$^{Val}$ Synthesis and ATP Consumption during the Overall tRNA$^{Val}$ Charging Process.*

The Val-tRNA$^{Val}$ synthesized was determined in 40-$\mu$L aliquots of the reaction mixture, containing [$^{14}$C]valine, as described above. The AMP formed was determined after the quenching of 10-$\mu$L aliquots of the reaction mixture, containing [$^{14}$C]ATP, with 20 $\mu$L of 15% acetic acid, centrifugation, and analysis of 10 $\mu$L of the supernatant by chromatography on silica gel sheets in the presence of methanol–water–ammoniac (6:2:1 v/v/v) solvent as described by Dietrich et al. (1976). The remaining ATP and the $PP_i$ released were determined after the quenching of 100-$\mu$L aliquots of the reaction mixture, containing [$\gamma$-$^{32}$P]ATP, in 300 $\mu$L of a suspension of acid-washed Norit (10% w/v in 7.5% perchloric acid) as described previously (Kern & Giegé, 1979).

*Val-tRNA$^{Val}$ Deacylation Kinetics.* The reaction mixtures contained, unless otherwise indicated, 50 mM NaHepes, pH 7.2, 20 mM $MgCl_2$, 10 mM AMP, 2 mM $PP_i$, enzyme as indicated, and either 3.6 $\mu$M [$^{14}$C]Val-tRNA$^{Val}$ (272 cpm/pmol) when the deacylation was tested or Val-[$^3$H]AtRNA$^{Val}$ when the rate of dissociation of tRNA$^{Val}$ from the synthetase was tested.

*Measurements of Rates of Dissociation of [$^{14}$C]aa-tRNA and [$^3$H]AtRNA from Valyl-tRNA Synthetase under Catalytic and Noncatalytic Conditions. Establishment of Stoichiometries of [$^{14}$C]Val~AMP and [$^{14}$C]Val-tRNA$^{Val}$ Bound to Valyl-tRNA Synthetase at the Steady State of tRNA$^{Val}$ Charging and of [$^3$H]AtRNA$^{Val}$ Bound on the Synthetase at the Steady State of the Reverse Reaction.* Aliquots (40 $\mu$L) of the incubation mixture were filtered through nitrocellulose disks. After being washed (3 times with 2 mL of a solution containing 10 mM NaMes pH 5.5, 2 mM $\beta$-mercaptoethanol, and 2 mM $MgCl_2$) and dried, the labeled material retained on the disks was determined by liquid scintillation. The experimental values were corrected for the yields of retention of the various complexes on the disks. These yields were established by comparing the amounts of the enzyme·ligand complexes retained on the nitrocellulose disks with the extents of saturation of the enzyme by these ligands after equilibrium gel filtration effected under the same conditions. Since under the experimental conditions used in this study (pH 5.5, 0 °C, and in the presence of the indicated substrates concentrations), the enzyme was found saturated 100% by Val~AMP and/or Val-tRNA$^{Val}$ by equilibrium gel filtration, the yields of retention of the various complexes on nitrocellulose disks were as follows: 100% for the enzyme·[$^{14}$C]Val-tRNA$^{Val}$ complex at either 2 or 20 mM $MgCl_2$ and in either the absence or presence of Val~AMP; 100% for the enzyme·[$^{14}$C]Val~AMP complex in the absence of Val-tRNA$^{Val}$ and at either 2 or 20 mM $MgCl_2$; 71% and 64% for the [$^{14}$C]Val~AMP-bound on the enzyme·Val-tRNA$^{Val}$ complex at 2 or 20 mM $MgCl_2$, respectively.

*Establishment of Stoichiometry of tRNA$^{Val}$ Bound to Valyl-tRNA Synthetase under Catalytic Conditions.* The reaction mixture contained 100 mM NaMes,[1] pH 5.5, 1 mM ATP, 1 mM L-valine, 2 mM $MgCl_2$, 40 $\mu$M [$^{32}$P]tRNA$^{Val}$ (42 cpm/pmol), and 3 $\mu$M enzyme. After 30 s–5 min of incubation at 0 °C, the amount of [$^{32}$P]tRNA$^{Val}$ bound to the enzyme (as free and charged tRNA) was determined by nitrocellulose disk filtration of 40-$\mu$L aliquots as described above.

## Results and Discussion

*Pre-Steady-State Kinetics of tRNA$^{Val}$ Charging.* The kinetics revealed the existence of a lag period during the early time, when the reaction was started with free enzyme (Figure 1A). The preincubation of the enzyme with ATP with or without magnesium did not affect this lag (Figure 1A), whereas its preincubation with tRNA$^{Val}$ or/and valine, respectively, decreased and suppressed it (Figure 1B). Valine

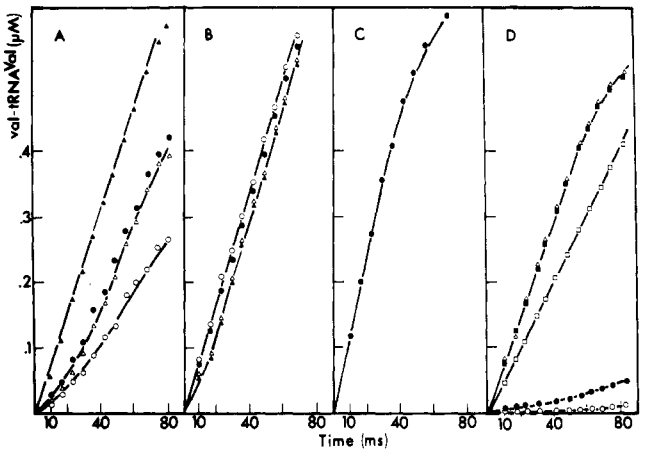


FIGURE 1: Pre-steady-state kinetics of tRNA$^{Val}$ charging when the reaction was started with various enzyme–substrate combinations. The reactions were conducted in the presence of either 50 mM NaHepes, pH 7.2 (A, B, and C) or 8.6 (D), or 50 mM NaMes, pH 6.0 (D), at 22 °C as described under Experimental Procedures. In (A) one syringe contained 1.84 $\mu$M enzyme, either no (○) or 20 mM (▲, ●, △) $MgCl_2$, either no (△, ○, ▲) or 5 mM (●) ATP, and either no (△, ●, ○) or 13 $\mu$M (▲) L-[$^{14}$C]valine. In (B) one syringe contained 1.84 $\mu$M enzyme, 20 mM $MgCl_2$, either no (△, ▲) or 100 M (○, ●) L-[$^{14}$C]valine, either no (○) or 10 $\mu$M (●, △, ▲) tRNA$^{Val}$, and either no (○, ●, △) or 5 mM (▲) ATP. In (C) one syringe contained 1.84 $\mu$M enzyme, 5 mM ATP, 20 mM $MgCl_2$, 10 $\mu$M L-[$^{14}$C]valine, and 10 units of inorganic pyrophosphatase/mL. In (D) the reactions were conducted at pH 6.0 (●, ○) or 8.6 (□, ■, △). One syringe contained 1.84 $\mu$M enzyme, 20 mM $MgCl_2$, either no (○, □, △) or 5 mM (●, ■) ATP, and either no (○, □) or 100 $\mu$M (●, ■, △) L-[$^{14}$C]valine. In each case the second syringe contained the various substrates at the necessary concentrations to ensure final concentrations as indicated under Experimental Procedures.

suppressed the lag even when present at a limiting concentration (i.e., 13 $\mu$M which corresponds to $K_D/4$; Figure 1A). When the reaction was started with preformed adenylate, no lag occurred, and the rate of transfer was optimal (Figure 1C). Finally, increasing pH decreased the amplitude of the lag and increased the valine effect (Figure 1).

In enzyme kinetics of lag generally reflects the establishment of the equilibrium of kinetically important steps preceding the catalysis. Consequently, valine activation or any other partial step preceding the transfer (such as the binding of substrates or changes in the state of the enzyme) could be responsible for the lag in the tRNA$^{Val}$ charging kinetic. To test this possibility, we investigated the [$\gamma$-$^{32}$P]ATP consumption during the first catalytic cycle of the enzyme. No lag was observed in these kinetics; in addition, the rate constants of valine activation were 230 and 250 $s^{-1}$ in the absence and in the presence of tRNA$^{Val}$, respectively (see Experimental Procedures). Since these values are considerably higher than the rate constant of transfer (12 $s^{-1}$), the lag observed in the tRNA$^{Val}$ charging cannot be related to the valine activation or to another step involved in this reaction; both steps occur independently.

*Steady-State Kinetics of tRNA$^{Val}$ Charging.* At pH 7.2 at 0 °C and in the presence of 30% glycerol, the steady-state kinetics of tRNA$^{Val}$ charging extrapolated back at $t = 0$ to a concentration of Val-tRNA$^{Val}$ which was equal to that of valyl-tRNA synthetase present (Figure 2A). At −20 °C (30% glycerol present), the amount of Val-tRNA$^{Val}$ synthesized did not exceed that of the enzyme present (Figure 2A). Finally, when the reaction was started with preformed enzyme·[$^3$H]-Val~AMP, ATP, [$^{14}$C]valine, and tRNA$^{Val}$, the [$^3$H]valine was incorporated much faster into the tRNA than the [$^{14}$C]valine (efficiency of the transfer, 90%, Figure 2B); the steady-state rate of tRNA$^{Val}$ charging was the same whether

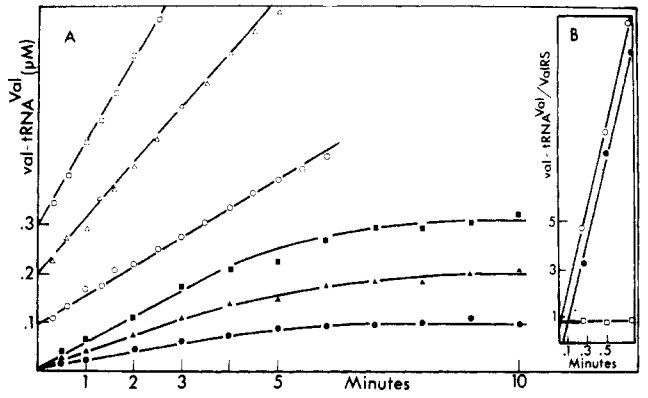


FIGURE 2: Kinetics of aminoacylation of tRNA$^{Val}$ in the presence of high concentrations of valyl-tRNA synthetase. (A) Starting from free enzyme. The incubation mixtures contained 50 mM NaHepes, pH 7.2, 5 mM ATP, 20 mM $MgCl_2$, 10 μM tRNA$^{Val}$, 100 μM L-[$^{14}$C]valine (195 cpm/pmol), 0.5 mM dithioerythritol, 30% glycerol, and 0.1 μM (○, ●), 0.2 μM (△, ▲), or 0.3 μM (□, ■) valyl-tRNA synthetase. The reactions were conducted either at 0 °C (○, △, □) or at −20 °C in an ethanol bath (●, ▲, ■), and the [$^{14}$C]Val-tRNA synthesized was determined as described under Experimental Procedures. (B) Starting from preformed enzyme·[$^3$H]valyladenylate. The incubation mixtures contained 50 mM NaHepes, pH 7.2, 0.1 μM enzyme·[$^3$H]valyladenylate (210 cpm/pmol), 0.5 mM dithioerythritol, 20 μM tRNA$^{Val}$, 20 mM $MgCl_2$, 5 mM ATP, and 100 μM L-valine, either $^{14}$C-labeled (195 cpm/pmol) (□, ●) or $^3$H-labeled (210 cpm/pmol) (○). The reactions were conducted at 0 °C. The (●) [$^{14}$C]- and (□, ○) [$^3$H]Val-tRNA synthesized was determined as described under Experimental Procedures.

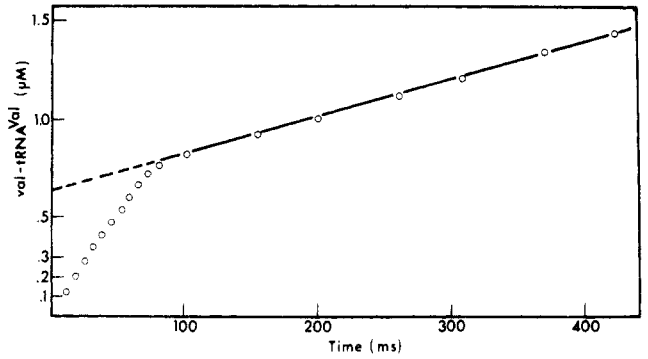


FIGURE 3: Biphasic tRNA$^{Val}$ aminoacylation kinetic. The kinetic was conducted by quenched flow at pH 7.2 and 22 °C. One syringe contained 1.84 μM enzyme, 10 μM L-[$^{14}$C]valine, 5 mM ATP, and 20 mM $MgCl_2$; the other syringe contained the substrates to ensure the final concentrations indicated under Experimental Procedures. The figure represents the combination of two kinetics: a first one performed from 0 to 80 ms and a second one from 0 to 400 ms.

the reaction was started with free enzyme or with preformed adenylate. When the reaction was conducted by rapid kinetics for a sufficiently long time to allow the completion of the first catalytic cycle of the enzyme, a biphasic kinetic was observed. After the first 100 ms, the rate of tRNA$^{Val}$ charging decreased sharply and equaled that measured by hand at the steady state (Figure 3). Finally, at pH 7.2 and 0 or 22 °C, the Val-tRNA$^{Val}$ synthesized in the burst was in a 0.6:1.0 stoichiometric ratio with the synthetase present (Figures 2B and 3). Table I shows that increasing pH and ionic strength increase the rate of tRNA$^{Val}$ charging more at the steady state than at the pre steady state. As a consequence the amplitudes of the bursts of Val-tRNA$^{Val}$, synthesis decreased with increasing pH and ionic strength.

The strong decrease of the rate of tRNA$^{Val}$ charging after completion of the first catalytic cycle of the synthetase suggests that one or several end products dissociate slowly from enzyme. This was tested by two different approaches. First, the concentrations of the enzyme·substrate and enzyme·end product complexes were determined at the steady state of the overall tRNA$^{Val}$ charging. Second, the dissociation of the various end products from enzyme was investigated directly.

Table I: Pre-Steady-State and Steady-State Rates of tRNA$^{Val}$ Charging as a Function of pH and KCl Concentrations[a]

| pH | KCl (mM) | rates ($s^{-1}$) pre steady state[b] | steady state[c] |
|---|---|---|---|
| 6.0 | 0 | 3.0 | 0.15 |
| 7.2 | 0 | 13.5 | 1.90 |
| | 100 | 4.5 | 4.50 |
| 8.6 | 0 | 8.4 | 3.40 |

[a] The rates were determined at 22 °C in the presence of saturating substrate concentrations as described under Experimental Procedures. [b] Determined by quenched-flow technique, starting from preformed enzyme·adenylate. [c] Determined by hand as described under Experimental Procedures.

Table II: Extents of Saturation of Valyl-tRNA Synthetase by Val~AMP and Val-tRNA$^{Val}$ at the Steady State of tRNA$^{Val}$ Charging at pH 5.5 and 0 °C[a]

| exptl conditions ATP (mM) | exptl conditions $MgCl_2$ (mM) | extent of saturation (%) of the synthetase by Val~AMP[b] | Val-tRNA$^{Val}$[c] |
|---|---|---|---|
| 1 | 2 | 100 | 100 |
| 1 | 20 | 90 | 100 |
| 0.02 | 2 | 40 | 100 |
| 0.02 | 20 | 60 | 60 |

[a] The incubation mixtures contained 100 mM NaMes, pH 5.5, ATP as indicated, 0.2 mM L-valine, $MgCl_2$ as indicated, 60 μM tRNA$^{Val}$, and 3 μM valyl-tRNA synthetase. After various incubation times from 30 s to 5 min at 0 °C (i.e., under initial rate conditions of tRNA charging) 40-μL aliquots were filtered through the nitrocellulose disks, and the enzyme-bound adenylate and aa-tRNA were determined as described under Experimental Procedures, taking into account their yields of retention of disks. [b] Incubation mixture contained α-$^{32}$P-labeled ATP (32 cpm/pmol) and unlabeled 0.2 mM L-valine. [c] Incubation mixture contained unlabeled ATP and $^{14}$C-labeled 0.2 mM L-valine (29 cpm/pmol).

*Extents of Saturation of Valyl-tRNA Synthetase by Val-tRNA$^{Val}$, tRNA$^{Val}$, and Val~AMP at the Steady State of tRNA$^{Val}$ Charging.* When all substrates were saturating, the synthetase was found saturated 100% by both Val-tRNA$^{Val}$ and Val~AMP at 2 mM magnesium and 100% by Val-tRNA$^{Val}$, and 90% by Val~AMP at 20 mM magnesium (Table II). In addition, the Val-tRNA$^{Val}$ synthesized and ATP consumed per enzyme before establishment of the steady state were in 1.0:2.0:1.0 and 0.95:1.9:1.0 stoichiometric ratios in the presence of 2 and 20 mM magnesium, respectively (Figure 4A). When ATP was limiting (20 μM), the other ligands being saturating, the synthetase was saturated 100% by Val-tRNA$^{Val}$ and 40% by Val~AMP at 2 mM magnesium and 60% by Val-tRNA$^{Val}$ and 60% by Val~AMP at 20 mM magnesium (Table II). In addition the Val-tRNA$^{Val}$ synthesized and ATP consumed per enzyme were in 1.0:1.4:1.0 and 0.5:1.2:1.0 stoichiometric ratios in the presence of 2 and 20 mM magnesium, respectively (Figure 4B). In the presence of 20 mM magnesium, the kinetics of tRNA$^{Val}$ charging and [γ-$^{32}$P]ATP consumption were both biphasic. This was also the case at 2 mM magnesium for the kinetics of tRNA$^{Val}$ charging, whereas those of [γ-$^{32}$P]ATP cleavage comprised three phases (Figure 4B): the first fast one, in which the ATP cleaved was stoichiometric with the synthetase present, was followed by a significantly slower phase during which 0.4 mol of ATP was consumed/mol of enzyme before establishment

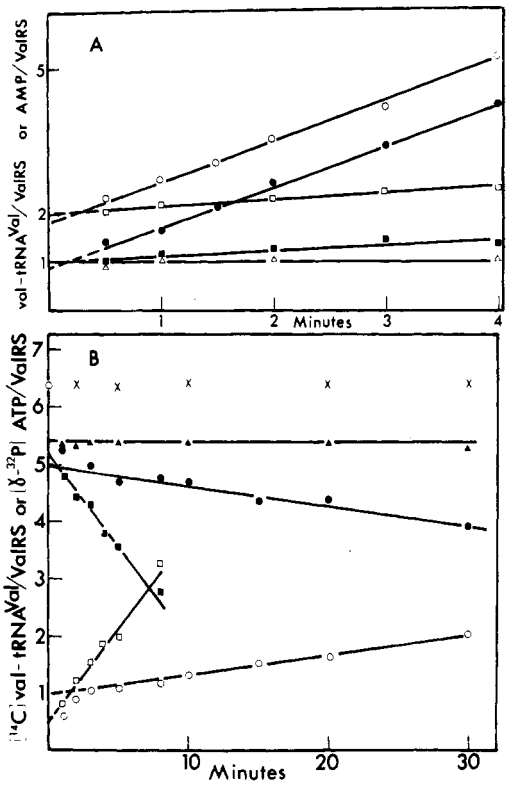


FIGURE 4: Parallel kinetics of $tRNA^{Val}$ charging and ATP consumption in the presence of high concentrations of valyl-tRNA synthetase. The reactions were conducted in 100 mM NaMes, pH 5.5, at 0 °C. (A) In the presence of saturating substrate concentrations. The reaction mixtures contained 2 mM (■, □) or 20 mM (●, ○) $MgCl_2$, 85 μM $tRNA^{Val}$, 0.2 mM L-valine, either $^{14}C$-labeled (29 cpm/pmol) (■, ●) or unlabeled (□, ○), 1 mM ATP, either $^{14}C$-labeled (21 cpm/pmol) (□, ○) or unlabeled (■, ●), 10 units of pyrophosphatase/mL, and 5 μM (■, ●) or 20 μM (□, ○) valyl-tRNA synthetase. The [$^{14}C$]-Val-tRNA synthesized (■, ●) and [$^{14}C$]AMP formed (□, ○) were determined as described under Experimental Procedures. In a control experiment (△) the [$^{14}C$]AMP formed by valyl-tRNA synthetase in the absence of $tRNA^{Val}$ was determined in the presence of 20 mM $MgCl_2$ and 0.2 mM valine. (B) In the presence of a limiting concentration of ATP. The incubation mixtures contained 2 mM (○, ●) or 20 mM (□, ■) $MgCl_2$, 0.2 mM L-valine, either $^{14}C$-labeled (29 cpm/pmol) (○, □) or unlabeled (●, ■), 19.2 μM ATP, either γ-$^{32}P$-labeled (132 cpm/pmol) (▲, ●) or unlabeled (○, □), 10 units of pyrophosphatase/mL, 3 μM valyl-tRNA synthetase, and 60 μM $tRNA^{Val}$. In two control experiments all reactants were present at the same concentrations ($MgCl_2$ at 20 mM) except $tRNA^{Val}$ (▲) or $tRNA^{Val}$ and valine (×). The [$^{14}C$]Val-tRNA synthesized (○, □) and [γ-$^{32}P$]ATP present (●, ■, ▲, ×) were determined as described under Experimental Procedures.

of the steady state (third phase). Finally, at the steady state the $tRNA^{Val}$ (either free or charged) was found associated to the synthetase in a 1.2:1.0 stoichiometric ratio (whereas only one $tRNA^{Val}$ or Val-$tRNA^{Val}$ binds to the free enzyme molecule).

*Dissociation of the End Products from Enzyme after the Catalytic Process.* At the steady state, $PP_i$ was incorporated into ATP ~20 times faster than $tRNA^{Val}$ was charged (rate constants 40 and 2 $s^{-1}$, respectively, at pH 7.2 and 22 °C). On the other hand, AMP dissociated much faster (100 and 5 times in the presence of 2 and 20 mM magnesium, respectively) than $tRNA^{Val}$ was charged (Table III). Under the same experimental conditions (pH 5.5 and 0 °C), the Val-$tRNA^{Val}$ generated by the transfer of valine from preformed adenylate to $tRNA^{Val}$ also dissociated much slower from enzyme than $tRNA^{Val}$ was charged at the steady state (3 and 12 times in the presence of 2 and 20 mM magnesium, respectively, Table III). However, the presence of the small ligands ATP and valine increased this rate significantly (2 and 12 times in the presence of 2 and 20 mM magnesium, respectively, Table III). Under steady-state conditions Val-$tRNA^{Val}$ dissociated from the enzyme at a rate close to that of $tRNA^{Val}$ charging ($10^{-3}$ and $1.2 \times 10^{-2}$ $s^{-1}$ in the presence of 2 and 20 mM magnesium, respectively).

The stimulation of the dissociation of the enzyme-bound Val-$tRNA^{Val}$ by ATP and valine was in fact promoted by Val~AMP and not by the small substrates even when together bound on the enzyme. Indeed, (1) when present individually, the small ligands did not affect the dissociation of Val-$tRNA^{Val}$ from enzyme, and (2) $PP_i$ reversed the stimulation of this dissociation promoted by the small substrates (Table III). Finally, Val~AMP promoted the dissociation of the enzyme-bound Val-$tRNA^{Val}$ only in the presence of a high concentration of free $tRNA^{Val}$ (Table III).

*Effect of Modification of Adenylate, tRNA, and aa-tRNA on Adenylate- and tRNA-Promoted Dissociation of Enzyme-Bound aa-tRNA.* This study was done by investigating (1) the effects of wrong adenylates (Thr~AMP and Ala~AMP) and of a nonspecific tRNA ($tRNA^{Phe}$) on the dissociation of the specific enzyme·Val-$tRNA^{Val}$ complex and (2) the effects of the specific substrates (Val~AMP and $tRNA^{Val}$) on the dissociation of nonspecific enzyme-bound aa-tRNAs (Val-$tRNA^{Phe}$ and Phe-$tRNA^{Val}$). Since valyl-tRNA synthetase does not activate phenylalanine and transfers the activated valine only slowly to $tRNA^{Phe}$ (D. Kern unpublished data), this study was mainly performed on complexes obtained by mixing the free reactants (enzyme or enzyme·adenylates and aa-tRNAs).

Table III shows that in the presence of Val~AMP, $tRNA^{Phe}$ did not promote the dissociation of the enzyme·Val-$tRNA^{Val}$ complex as efficiently than $tRNA^{Val}$ did. On the other hand, whereas the dissociation kinetics of the enzyme·Val-$tRNA^{Val}$ and enzyme·$tRNA^{Val}$ complexes generated by catalysis (respectively by transfer of valine from Val~AMP to $tRNA^{Val}$ and by deacylation of Val-$tRNA^{Val}$ in the presence of AMP and $PP_i$) were both of the first order (results not shown), those of the complexes generated by mixing the free reactants (enzyme and Val-$tRNA^{Val}$ or $tRNA^{Val}$) were biphasic (Figure 5): one part of the complex (60% of the enzyme·Val-$tRNA^{Val}$ and 40% of the enzyme·$tRNA^{Val}$ in the presence of 20 mM magnesium) dissociated much faster than the second part. Increasing magnesium concentrations increased the amplitude of the first (fast) phase and the rate of dissociation of the second (slow) phase of the kinetics (Figure 5). Whereas Val~AMP increased the rate of the second slow phase of dissociation of the enzyme·Val-$tRNA^{Val}$ complex (2 and 80 times in the presence of 2 and 20 mM magnesium, respectively, the rate constants being $2.3 \times 10^{-4}$ and $9.8 \times 10^{-4}$ $s^{-1}$ at 2 mM magnesium and $5.9 \times 10^{-4}$ and $4.6 \times 10^{-2}$ $s^{-1}$ at 20 mM magnesium in the absence and in the presence of adenylate, respectively), it did not affect the dissociation of the enzyme·$tRNA^{Val}$ complex at 2 or 20 mM magnesium whether this complex was generated by catalysis or by mixing the free reactants (Figure 5). This indicates that an homogeneous type of complex is generated by the catalysis whereas two types of complexes differing by their dissociation rate constants are generated by mixing the free reactants.

The dissociation of the enzyme-bound Val-$tRNA^{Phe}$ was monophasic and occurred fast even in the presence of 2 mM magnesium either in the absence or in the presence of adenylate (Figure 5). On the contrary, the dissociation of the enzyme-bound Phe-$tRNA^{Val}$ was biphasic; the amplitude of the first (fast) phase was increased as compared to that observed in the dissociation of Val-$tRNA^{Val}$ (Figure 5); in ad-

Table III: First-Order Rate Constants ($k$) of Dissociation of End Products from Enzyme after the Catalytic Process under Various Conditions[a]

| expt | end product | ligands present | | | | | $k$ ($\times 10^2$ $s^{-1}$) |
|---|---|---|---|---|---|---|---|
| | | $MgCl_2$ (mM) | tRNA ($\mu$M) | ATP (mM) | Val (mM) | $PP_i$ (mM) | |
| 1 | AMP | 2 | 30 | | | | 10 |
| 2 | AMP | 20 | 30 | | | | 5 |
| 3 | Val-$tRNA^{Val}$ | 2 | 30 | | | | 0.038 |
| 4 | Val-$tRNA^{Val}$ | 20 | 30 | | | | 0.096 |
| 5 | Val-$tRNA^{Val}$ | 2 | 30 | 1 | 1 | | 0.080 |
| 6 | Val-$tRNA^{Val}$ | 20 | 30 | 1 | 1 | | 1.0 |
| 7 | Val-$tRNA^{Val}$ | 20 | 30 | 1 | | | 0.096 |
| 8 | Val-$tRNA^{Val}$ | 20 | 30 | | 1 | | 0.096 |
| 9 | Val-$tRNA^{Val}$ | 20 | 30 | 1 | 1 | 1 | 0.096 |
| 10 | Val-$tRNA^{Val}$ | 20 | 0.1 | 1 | 1 | | 0.096 |
| 11 | Val-$tRNA^{Val}$ | 20 | 30 | 1 | 1 | | 0.30 |

[a] The reaction mixtures (total volume, 400 $\mu$L) contained 100 mM NaMes, pH 5.5, $MgCl_2$ as indicated, 0.4 $\mu$M valyl-tRNA synthetase·Val~AMP complex either [$^{32}$P]AMP-labeled (in experiments 1 and 2) or [$^{14}$C]Val-labeled (in experiments 3–11), and except in experiment 9, 10 units of inorganic pyrophosphatase per mL. In experiments 1–9, 30 $\mu$M $tRNA^{Val}$ was added, and after 30 s of incubation at 0 °C (to ensure the transfer of valine to $tRNA^{Val}$) the small ligands (ATP, valine, and $PP_i$) were added at the indicated concentrations. In experiment 10, after addition of 30 $\mu$M $tRNA^{Val}$ and incubation for 30 s, the mixture was diluted in 100 mL of NaMes, pH 5.5, containing the various ligands at the indicated concentrations. In experiment 11, 0.5 $\mu$M $tRNA^{Val}$ was added to the reaction mixture and after 30 s of incubation, $tRNA^{Phe}$, ATP, and valine were added at the indicated concentrations. The dissociations of the [$^{14}$C]Val-$tRNA^{Val}$·enzyme complexes were followed at 0 °C by nitrocellulose disk filtrations of aliquots as described under Experimental Procedures. It was verified that under the experimental conditions used no appreciable deacylation of Val-$tRNA^{Val}$ occurred. The steady-state rate constants of $tRNA^{Val}$ charging were $10^{-3}$ and $1.2 \times 10^{-2}$ $s^{-1}$ in the presence of 2 and 20 mM $MgCl_2$, respectively.

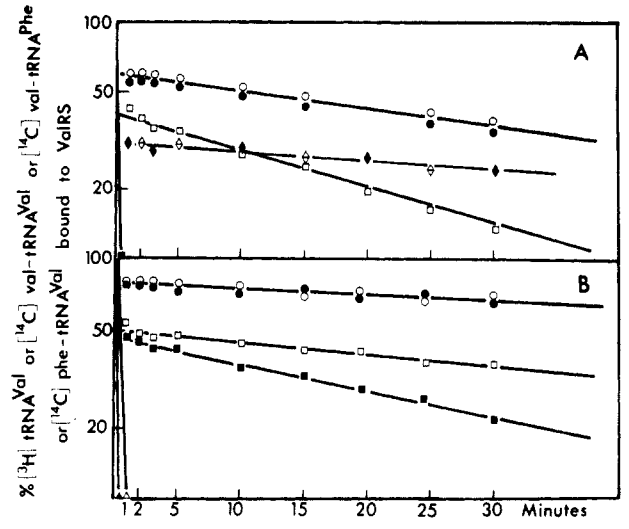


FIGURE 5: Kinetics of dissociation of various valyl-tRNA synthetase-bound tRNAs and aa-tRNAs under various conditions in the presence of 20 mM (A) or 2 mM (B) magnesium. The incubation mixtures (total volume, 400 $\mu$L) contained 100 mM NaMes, pH 5.5, 3 $\mu$M valyl-tRNA synthetase, either 3 $\mu$M periodate-oxidized [$^3$H]At$RNA^{Val}$ (39 cpm/pmol) (○, ●), [$^{14}$C]Val-$tRNA^{Val}$ (272 cpm/pmol) (□, ■), [$^{14}$C]Phe-$tRNA^{Val}$ (122 cpm/pmol) (◇, ◆), or [$^{14}$C]Val-$tRNA^{Phe}$ (135 cpm/pmol) (△, ▲), and 10 units of inorganic pyrophosphatase/mL. After incubation for 1 min at 0 °C, the dissociations were followed either in the absence of ATP and valine (○, □, ◇, △) or in the presence of 1 mM ATP plus 1 mM valine (●, ■, ◆, ▲) and in the presence of 60 $\mu$M $tRNA^{Val}$ (○, ●, □, ■, ◇, ◆) or $tRNA^{Phe}$ (△, ▲) as described under Experimental Procedures. It was verified that the aa-tRNAs were not significantly deacylated under the experimental conditions used.

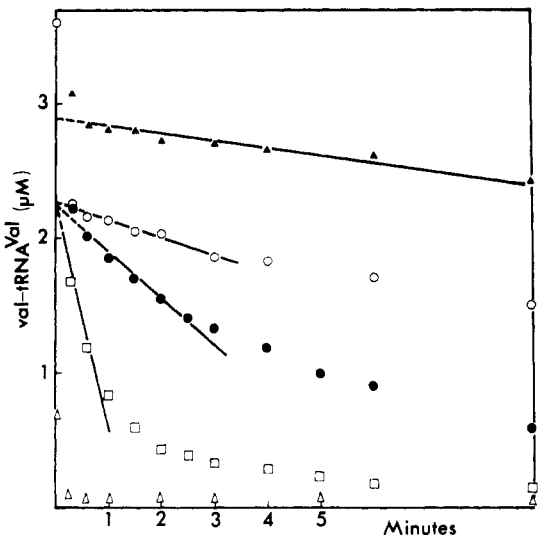


FIGURE 6: Kinetics of deacylation of Val-$tRNA^{Val}$ (AMP and $PP_i$ dependent) in the presence of high concentrations of valyl-tRNA synthetase (▲, ○, ●, □). Extent of saturation of the synthetase by Val-$tRNA^{Val}$ at the steady state of the deacylation reaction (△). The incubation mixtures contained 50 mM NaHepes, pH 7.2, 20 mM $MgCl_2$, 10 mM AMP, 2 mM $PP_i$, 3.6 $\mu$M [$^{14}$C]Val-$tRNA^{Val}$ (272 cpm/pmol), and either 0.7 $\mu$M (▲, △) or 1.3 $\mu$M (○, ●, □) valyl-tRNA synthetase. The reactions were conducted either at 0 (▲, ○, △), at 10 (●), or at 20 (□) °C. The total [$^{14}$C]Val-$tRNA^{Val}$ was determined by trichloroacetic acid precipitation and the enzyme-bound [$^{14}$C]Val-$tRNA^{Val}$ by nitrocellulose disk filtration as described under Experimental Procedures. The experimental values of deacylation were corrected for the chemical and enzymatic deacylations (not AMP and $PP_i$ dependent) which were estimated in control experiments conducted in the absence of AMP and $PP_i$. The experimental values of enzyme-bound aa-tRNA were corrected for the yield of retention of the complex on the disks (87% under the experimental conditions).

dition, Val~AMP did not affect this dissociation (Figure 5). Finally, Thr~AMP and Ala~AMP were unable to promote the dissociation of the enzyme-bound Val-$tRNA^{Val}$ as efficiently as Val~AMP did; indeed, in the presence of 20 mM magnesium the wrong adenylates increased the dissociation rate of the complex by a factor of 2 whereas Val~AMP increased it 12 times.

*Kinetics of Deacylation of Val-$tRNA^{Val}$ by Valyl-tRNA Synthetase in the Presence of AMP and $PP_i$.* At pH 7.2 and either 0, 10, or 20 °C, the steady-state kinetics of the reversal of $tRNA^{Val}$ charging extrapolated back at $t = 0$ to bursts in which the deacylated $tRNA^{Val}$ was in a 1:1 stoichiometric ratio with the synthetase present (Figure 6); thus, a strong decrease in the reaction rate occurs after completion of the first catalytic cycle of the enzyme. End product complex measurements performed at the steady state showed that the synthetase was poorly saturated by Val-$tRNA^{Val}$ (Figure 6) but mainly by uncharged $tRNA^{Val}$; in addition, no Val~AMP was found associated to the enzyme. Finally, the first-order rate constant of dissociation of the enzyme-bound $tRNA^{Val}$ equaled the

steady-state rate of AMP- and $PP_i$-dependent Val-$tRNA^{Val}$ deacylation ($3 \times 10^{-4}$ $s^{-1}$ at pH 5.5 and 0 °C and in the presence of 20 mM magnesium).

## General Discussion

The kinetics reported here display several partial steps at the pre steady state and at the steady state of the overall $tRNA^{Val}$ charging process which can be rate determining.

*Rate-Determining Step of $tRNA^{Val}$ Charging at Pre Steady State.* The lag period observed in the early time of $tRNA^{Val}$ charging corresponds to a step which occurs independently upon the valine activation, contrary to that reported for the yeast arginylation system (Fersht et al., 1978) and other aminoacylation systems (Lagerkvist et al., 1977). It can reasonably be assumed that it reflects a conformational change necessary for the transfer of valine to $tRNA^{Val}$ and which can be induced with increasing efficiencies by $tRNA^{Val}$, valine, and Val~AMP, respectively. Two major possibilities can be proposed to explain the particular kinetic behavior of valyl-tRNA synthetase at the pre steady state: (1) the substrates $tRNA^{Val}$, valine, and Val~AMP generate the active site of the transfer on the synthetase with increasing efficiencies (eq 2)

$$\begin{gathered} E + tRNA^{Val} \rightarrow E_1 \cdot tRNA^{Val} \\ E + Val \rightarrow E_2 \cdot Val \\ E + Val + ATP \cdot MgCl_2 \rightarrow E_3 \cdot AMP \sim Val \end{gathered} \quad (2)$$

E being inactive, $E_1$ and $E_2$ partially active, and $E_3$ fully active for the transfer; (2) the catalytically functional interaction of $tRNA^{Val}$ with the synthetase results from a multistep process involving several conformational transitions which are in equilibrium (eq 3)

$$E + tRNA^{Val} \rightleftharpoons (E \cdot tRNA^{Val})_1 \rightleftharpoons (E \cdot tRNA^{Val})_2 \rightleftharpoons (E \cdot tRNA^{Val})_3 \quad (3)$$

$(E \cdot tRNA^{Val})_1$ and $(E \cdot tRNA^{Val})_3$ being respectively inactive and fully active for the transfer; valine and Val~AMP generate enzyme forms (E* and E**, respectively) displacing this equilibrium toward the active complex with increasing efficiencies (eq 4):

$$E + tRNA^{Val} \rightleftharpoons (E \cdot tRNA^{Val})_1 \rightleftharpoons (E \cdot tRNA^{Val})_3$$

$$E + Val \rightarrow E^* \cdot Val \overset{+tRNA^{Val}}{\rightleftharpoons} (E^* \cdot Val \cdot tRNA^{Val})_2 \rightleftharpoons (E^* \cdot Val \cdot tRNA^{Val})_3 \quad (4)$$

$$E + Val + ATP \cdot MgCl_2 \rightarrow E^{**} \cdot Val \sim AMP \overset{+tRNA}{\rightleftharpoons} (E^{**} \cdot Val \sim AMP \cdot tRNA^{Val})_3$$

Since the valine effect occurs even when valine is present at a limiting concentration, the E* form does not reverse instantaneously toward the E form after dissociation of the valine as shown in eq 5. This effect resembles the mnemonic effects

$$\begin{gathered} E^* \cdot Val \xrightarrow{fast} E + Val \\ E^* + tRNA^{Val} \xrightarrow{fast} (E^* \cdot tRNA^{Val})_2 \\ E^* \xrightarrow{slow} E \end{gathered} \quad (5)$$

induced by modified substrates on some enzymes of the metabolic pathway and which consist of an irreversible transconformation of these enzymes during their first catalytic cycle (Ricard et al., 1974; Meunier et al., 1974).

One can now speculate which of the two mechanisms (eq 2 or eq 3 and 4) is most prevalent in the catalytic process of $tRNA^{Val}$ charging. Using other approaches, Von der Haar & Cramer (1978a,b) brought convincing arguments indicating that in the yeast valylation and in other aminoacylation systems, the productive interactions of the tRNAs with their cognate synthetases occur in a multistep process; on the other hand, strong tRNA effects on the kinetic parameters of the $PP_i$–ATP exchange have been reported for a large number of tRNA charging systems [i.e., Mitra & Mehler (1966), Parfait & Grosjean (1972), Di Natale et al. (1974), Nazario & Evans (1974), Von der Haar & Gaertner (1975), Jacques & Blanquet (1977), Wehmeyer et al. (1979), Kern & Giegé (1979), Godeau (1980), and Kern & Lapointe (1980)], indicating an interrelation between the binding sites of adenylate and tRNA. These facts strongly support the second mechanism (eq 3 and 4) in the yeast valylation system; it could be that the lag reflects the productive interaction of the amino acid acceptor end of the tRNA near the adenylate. Although the affinity of the synthetase for $tRNA^{Val}$ is the highest at acidic pH (Bonnet & Ebel, 1975), this interaction occurs more readily at alkaline pH.

*Mechanism of Dissociation of Val-$tRNA^{Val}$ from Valyl-tRNA Synthetase.* We bring evidence that at the steady state of $tRNA^{Val}$ charging the dissociation of the nascent Val-$tRNA^{Val}$ from enzyme is promoted by the newly synthesized Val~AMP as shown in eq 6. A similar mechanism had first

$$\begin{gathered} E \cdot Val\text{-}tRNA^{Val} \xrightarrow{slow} E + Val\text{-}tRNA^{Val} \\ E \cdot Val\text{-}tRNA^{Val} + ATP \cdot MgCl_2 + Val \xrightarrow{fast} \\ E \cdot AMP \sim Val \cdot Val\text{-}tRNA^{Val} \xrightarrow{fast} \\ E \cdot AMP \sim Val + Val\text{-}tRNA^{Val} \end{gathered} \quad (6)$$

been proposed by Yarus & Berg (1969) and by Eldred & Schimmel (1972) for the *E. coli* isoleucylation system. Additional steps involved in the overall yeast valylation system were detected by studying the dissociation of the enzyme·Val-$tRNA^{Val}$ complex generated by mixing the free reactants (enzyme and Val-$tRNA^{Val}$).

*(1) Dissociation of Enzyme·Val-$tRNA^{Val}$ Complex Generated by Mixing Free Reactants.* The biphasic dissociation kinetics indicate that two types of complexes [$(E \cdot Val\text{-}tRNA^{Val})_1$ and $(E \cdot Val\text{-}tRNA^{Val})_2$] are formed whose dissociation rate constants exhibit different dependences upon the presence of adenylate and free $tRNA^{Val}$: the $(E \cdot Val\text{-}tRNA^{Val})_1$ complex dissociates fast in the presence of an excess of free $tRNA^{Val}$ even when adenylate is absent, whereas the $(E \cdot Val\text{-}tRNA^{Val})_2$ complex dissociates fast in the presence of both adenylate and free $tRNA^{Val}$. It is likely that both complexes are in equilibrium (eq 7):

$$(E \cdot Val\text{-}tRNA^{Val})_1 \rightleftharpoons (E \cdot Val\text{-}tRNA^{Val})_2 \quad (7)$$

This equilibrium is rapidly established since both complexes are present in the same ratio whatever the incubation time. In addition, since under the experimental conditions used, both complexes are present in a ratio near to 1, their dissociation constants are similar. This is consistent with the fact that an homogeneous $K_D$ value could be determined for this system (Kern et al., 1980). When present individually, magnesium and adenylate displace slightly the equilibrium toward the $(E \cdot Val\text{-}tRNA^{Val})_1$ complex, whereas when present together, they displace it strongly. This is probably due to a synergistic effect. As a consequence the stimulatory effect of adenylate, magnesium, and $tRNA^{Val}$ on the dissociation of the enzyme·Val-$tRNA^{Val}$ complex seems to result from the displacement

Scheme I: Dissociation Steps of End Product Complex[a]

$$E\cdot AMP\cdot PP_i\cdot Val\text{-}tRNA^{Val} \xrightarrow[(1)]{k_1} E\cdot Val\text{-}tRNA^{Val} + AMP + PP_i$$

(2) $k_2$ + ATP·$MgCl_2$

$$E\cdot ATP\cdot MgCl_2\cdot Val\text{-}tRNA^{Val}$$

(3) $k_3$

$$E\cdot ATP\cdot MgCl_2\cdot (Val\text{-})tRNA^{Val}$$

(4) $k_4$ + Val

$$E\cdot AMP\sim Val\cdot (Val\text{-})tRNA^{Val} + PP_i\cdot MgCl_2$$

(5) $k_5$

$$[E\cdot AMP\sim Val\cdot (Val\text{-})tRNA^{Val}]_2$$

(6) $k_6$

$$[E\cdot AMP\sim Val\cdot (Val\text{-})tRNA^{Val}]_1$$

(7) $k_7$

$$[E\cdot AMP\sim Val\cdot (Val\text{-})tRNA^{Val}\cdot tRNA^{Val}]_1$$

(8) $k_8$

$$E\cdot AMP\sim Val\cdot tRNA^{Val} + Val\text{-}tRNA^{Val}$$

[a] Val-$tRNA^{Val}$ and (Val-)$tRNA^{Val}$ represent respectively the forms of the complexes in which the valine bound to $tRNA^{Val}$ is or is not in the adenylate site.

by adenylate and magnesium of equilibrium 7 toward the $(E\cdot Val\text{-}tRNA^{Val})_1$ complex, which then dissociates fast in the presence of free $tRNA^{Val}$.

*(2) Dissociation of Val-$tRNA^{Val}$ from Enzyme after the Catalytic Step.* The kinetic studies reported in this work allow us to propose following steps after the transfer reaction (Scheme I). Step 1 is the fast dissociation of AMP and $PP_i$ (see Results). Steps 2 and 3 are the liberation of the amino acid site. This occurs after binding of ATP·$MgCl_2$ (step 2), by ejection of the amino acid moiety of Val-$tRNA^{Val}$ from adenylate site or translocation to another site (step 3). These steps were visualized by ATP consumption measurements when the $tRNA^{Val}$ charging was conducted in the presence of low ATP and magnesium concentrations (Figure 4B). Under these conditions the adenylate is much more slowly synthesized after the transfer step (when the enzyme is saturated by Val-$tRNA^{Val}$) than before the first catalytic cycle of the transfer (when the enzyme is saturated by uncharged $tRNA^{Val}$). Since it was shown that $tRNA^{Val}$ and Val-$tRNA^{Val}$ affect similarly the rate of adenylate synthesis (Kern & Giegé, 1979), this kinetic behavior cannot be related to a differential allosteric effect of the enzyme-bound Val-$tRNA^{Val}$ and $tRNA^{Val}$ on the adenylation site. Thus, it can reasonably be assumed that the slow rate of adenylate synthesis, after completion of the first catalytic cycle of $tRNA^{Val}$ charging, reflects the slow dissociation of the amino acid moiety of Val-$tRNA^{Val}$ from the adenylate site before synthesis of a new adenylate molecule. ATP and magnesium promote this step probably by increasing the dynamicity of the amino acid moiety of Val-$tRNA^{Val}$, thus favoring its ejection from adenylate site. Step 4 is the binding of valine and the synthesis of a new adenylate molecule. Step 5 is the appearance of the $(E\cdot AMP\sim Val\cdot Val\text{-}tRNA^{Val})_2$ complex. This emerges from the fact that the catalysis generates an homogeneous type of complex which exhibits properties similar to those of the $(E\cdot Val\text{-}tRNA^{Val})_2$ one generated by mixing the free reactants: the complex dissociates according to a first-order reaction, which is promoted by magnesium, adenylate, and $tRNA^{Val}$ together. However, since in the presence of 20 mM magnesium, adenylate promotes more readily the dissociation of Val-$tRNA^{Val}$ from the $(E\cdot AMP\sim Val\cdot Val\text{-}tRNA^{Val})_2$ complex than from the native one generated by the transfer, it must be assumed that the native $E\cdot Val\text{-}tRNA^{Val}$ complex must transconform into the $(E\cdot Val\text{-}tRNA^{Val})_2$ one. This transconformation is promoted by both the ligands adenylate and magnesium, since at low magnesium concentration (2 mM) and in the presence of adenylate both complexes, the native one and the $(E\cdot Val\text{-}tRNA^{Val})_2$ one, dissociate at a similar rate. The further steps are those detected in the dissociation of the complex generated by mixing the free reactants. Step 6 is the displacement of the equilibrium from the $(E\cdot AMP\sim Val\cdot Val\text{-}tRNA^{Val})_2$ complex toward the $(E\cdot AMP\sim Val\cdot Val\text{-}tRNA^{Val})_1$ one able to dissociate in the presence of free $tRNA^{Val}$. Adenylate promotes this shift. Steps 7 and 8 are the exchange between the enzyme-bound nascent Val-$tRNA^{Val}$ and free $tRNA^{Val}$. Since this step is promoted by a high concentration of free $tRNA^{Val}$ and since 15–20% of the enzyme molecules are saturated by two tRNAs at the steady state of $tRNA^{Val}$ charging (probably one as Val-$tRNA^{Val}$ and another one as uncharged $tRNA^{Val}$), the sequence of the partial steps involves an enzyme form able to bind a new $tRNA^{Val}$ molecule (step 7) before dissociation of the newly synthesized Val-$tRNA^{Val}$ (step 8).

*Rate-Determining Step of $tRNA^{Val}$ Charging at Steady State.* Variations in the rate-determining step were observed depending upon the experimental conditions. Since in the presence of saturating substrate concentrations the synthetase is saturated ~100% by Val-$tRNA^{Val}$ and Val~AMP, the rate-determining step follows the synthesis of the adenylate on the end product complex. However, this step is not the same in the presence of 20 or 2 mM magnesium. In the presence of 20 mM magnesium, adenylate promotes more readily the dissociation of the $(E\cdot Val\text{-}tRNA^{Val})_2$ complex than the native one generated by the transfer, indicating that step 5 is probably rate determining. In addition, the accumulation of a small amount of $E\cdot AMP\sim Val\cdot Val\text{-}tRNA^{Val}\cdot tRNA^{Val}$ complex at the steady state indicates that the rate constants in Scheme I are in following relationship: $k_1, k_2, k_3, k_4, k_6, k_7 \gg k_8 > k_5$. In the presence of 2 mM magnesium, the end product complex dissociates at the same rate whether it is generated by catalysis or by mixing the free reactants, indicating that step 6 is rate determining: $k_1, k_2, k_3, k_4, k_5, k_7 \gg k_8 > k_6$. In the presence of a limiting ATP and a low magnesium concentration (20 $\mu$M and 2 mM, respectively), the synthetase is saturated 100% by Val-$tRNA^{Val}$ and 40% by Val~AMP at the steady state. Thus, in addition to step 6 (which is rate determining in the presence of high ATP and magnesium concentrations), step 3 also becomes partially rate determining: $k_1, k_2, k_4, k_5, k_7, k_8 \gg k_3, k_6$. Finally, when ATP is limiting (20 $\mu$M) and magnesium present at a high concentration (20 mM), the synthetase is saturated 60% by Val-$tRNA^{Val}$ and 60% by Val~AMP, indicating that in addition to step 5 (which is rate determining when ATP and magnesium are both present at high concentrations), the adenylate synthesis (in the first catalytic cycle as well as at the steady state i.e., step 4) becomes partially rate determining: $k_1, k_2, k_3, k_6, k_7, k_8 \gg k_4, k_5$.

*Rate-Determining Step in Reversal of $tRNA^{Val}$ Charging.* The accumulation of the enzyme·$tRNA^{Val}$ complex at the steady state indicates that the $tRNA^{Val}$ generated after the fast deacylation of Val-$tRNA^{Val}$ (AMP dependent) and the

fast pyrophosphorolysis of the adenylate dissociates slowly from enzyme. This is confirmed by the equality of the steady-state rate of the reversal of $tRNA^{Val}$ charging and the rate of dissociation of the enzyme-bound $tRNA^{Val}$. We thus propose the following scheme for the reversal of $tRNA^{Val}$ charging (eq 8):

$$\begin{aligned} E + Val\text{-}tRNA^{Val} + AMP + PP_i &\xrightarrow{fast} E\cdot AMP\cdot PP_i\cdot Val\text{-}tRNA^{Val} \\ &\xrightarrow{fast} E\cdot AMP\sim Val\cdot PP_i\cdot Val\text{-}tRNA^{Val} \\ &\xrightarrow{fast} E\cdot ATP\cdot Val\cdot tRNA^{Val} \\ &\xrightarrow{fast} E\cdot tRNA^{Val} + ATP + Val \\ &\xrightarrow{slow} E + tRNA^{Val} \end{aligned} \quad (8)$$

*Nature of the Two Complexes $(E\cdot Val\text{-}tRNA^{Val})_1$ and $(E\cdot Val\text{-}tRNA^{Val})_2$.* The two complexes are generated in equivalent parts by mixing free enzyme with $Val\text{-}tRNA^{Val}$. Since they are also generated when uncharged $tRNA^{Val}$ is mixed with the synthetase, the equilibrium being, however, in this case slightly displaced toward the $(E\cdot tRNA^{Val})_2$ complex, both types of complexes differ by the nature of the interaction of the $Val\text{-}tRNA^{Val}$ (or $tRNA^{Val}$) with the synthetase: the $(E\cdot Val\text{-}tRNA^{Val})_1$ complex involves directly or indirectly the amino acid moiety and the amino acid acceptor stem of the aa-tRNA, whereas the $(E\cdot Val\text{-}tRNA^{Val})_2$ complex involves rather other regions of the aa-tRNA. This is consistent with the dissociation behavior of $Val\text{-}tRNA^{Phe}$ from the synthetase; indeed, the substitution of the $tRNA^{Val}$ moiety of $Val\text{-}tRNA^{Val}$ by $tRNA^{Phe}$ results in a strong displacement of equilibrium 7 toward the $(E\cdot aa\text{-}tRNA)_1$ complex. However, this model would predict a displacement of equilibrium 7 toward the $(E\cdot aa\text{-}tRNA)_2$ complex after substitution of the valine moiety of $Val\text{-}tRNA^{Val}$ by phenylalanine. This does not occur. Since it was shown that the tRNAs are modified in their conformation after aminoacylation (Caron et al., 1976), this result would indicate that the $tRNA^{Val}$ is in another conformational state whether it is charged with valine or phenylalanine, resulting in a modification of its interacting properties with the synthetase. Such model of interaction of the aa-tRNA with the synthetase according to two modes implying either the amino acid moiety and the amino acid acceptor stem or other regions of the aa-tRNA has first been proposed by Pimmer & Holler (1979) for the *E. coli* phenylalanine system.

*Specificity of Adenylate-Promoted Dissociation of the Enzyme-Bound $Val\text{-}tRNA^{Val}$.* Since adenylate stimulates the dissociation of the enzyme-bound $Val\text{-}tRNA^{Val}$ and is without effect when $tRNA^{Val}$ is not charged or charged with phenylalanine, it appears that the valine moiety of $Val\text{-}tRNA^{Val}$ is involved in this mechanism. The $tRNA^{Val}$ moiety of $Val\text{-}tRNA^{Val}$ seems also to be involved since its substitution by $tRNA^{Phe}$ affects the dissociation of the enzyme-bound aa-tRNA. Finally, since wrong adenylates Thr∼AMP and Ala∼AMP are much less efficient than Val∼AMP in promoting the dissociation of the enzyme-bound $Val\text{-}tRNA^{Val}$, the valine moiety of the adenylate seems also to be involved in this mechanism of dissociation. This indicates that the mechanism of dissociation of the nascent $Val\text{-}tRNA^{Val}$ promoted by Val∼AMP is highly specific for the ligands of the synthetase.

*Stoichiometry of Substrate Binding Sites on Yeast Valyl-tRNA Synthetase.* The involvement of both amino acid moieties (that of $Val\text{-}tRNA^{Val}$ and that of Val∼AMP) in the adenylate-promoted dissociation of the enzyme-bound $Val\text{-}tRNA^{Val}$ strongly suggests the existence of two binding sites for valine on the synthetase. Since only one site could be detected on the free enzyme, it must be assumed that a second valine site, highly specific for the valine moiety of $Val\text{-}tRNA^{Val}$, is generated during the catalytic process. However, it could also be that the valine moiety of $Val\text{-}tRNA^{Val}$ is not bound on a specific site and that the adenylate-promoted dissociation of $Val\text{-}tRNA^{Val}$ involves a particular conformational state of the enzyme·AMP∼Val-$Val\text{-}tRNA^{Val}$ complex.

Our study also shows that the end product dissociation process generates an enzyme form able to bind two tRNAs (probably an uncharged $tRNA^{Val}$ in addition to the newly synthesized $Val\text{-}tRNA^{Val}$). However, it cannot be concluded whether the two tRNAs are bound on different regions of the enzyme or if they interact on the same binding site. Since only one tRNA (or aa-tRNA) binds on the free enzyme, our results could indicate that the enzyme possesses two strongly anticooperative tRNA binding sites which become less anticooperative in one of the enzyme forms involved in the catalytic process. However, the results would also agree with the existence of one tRNA interacting site assuming that before complete dissociation of the nascent $Val\text{-}tRNA^{Val}$ a new $tRNA^{Val}$ molecule can bind to the synthetase.

Valyl-tRNA synthetase belongs to the family of monomeric synthetases of molecular weight ∼100 000, which contain a large part of sequence duplication (Kern et al., 1975). For some of these synthetases the existence of two distinct domains has been demonstrated (Waterson et al., 1979). However, there is no information about their function, and, in particular, it is not known whether these domains can be related to the existence of two active sites. Evidence for the existence of two substrate binding sites on some of these enzymes has been brought forth. Fersht (1975) showed that the valyl-tRNA synthetase from *E. coli* binds two amino acids and postulated the existence of two adenylate sites on this monomeric synthetase. On the basis of inhibition experiments of $tRNA^{Ile}$ charging by yeast isoleucyl-tRNA synthetase in the presence of tRNAs modified in their CCA end, Von der Haar & Cramer (1978a) postulated the existence of two binding sites for $tRNA^{Ile}$ on this synthetase. Finally, proofreading and deacylation experiments (not AMP and $PP_i$ dependent) also suggest the existence of a specific site for the tRNA-bound amino acid (Fersht & Kaethner, 1976; Bonnet, 1974). We bring some arguments for the existence of two binding sites for $tRNA^{Val}$ and valine on the monomeric valyl-tRNA synthetase from baker's yeast. However, further investigations are necessary to fully establish their existence.

*Conclusion.* The present work brings strong evidence that conformational changes occur on valyl-tRNA synthetase during the catalytic process of $tRNA^{Val}$ charging. The conformational transitions occurring before the transfer step consist probably in an adequate positioning of the amino acid acceptor end of tRNA near the valine moiety of Val∼AMP. We show that the small ligands contribute largely in these changes. After the transfer, the newly synthesized $Val\text{-}tRNA^{Val}$ dissociates from synthetase via a multistep process: after ejection of the valine moiety of $Val\text{-}tRNA^{Val}$ from the adenylate site by $ATP\cdot MgCl_2$, a new adenylate molecule is synthesized which promotes then in the presence of a high concentration of free $tRNA^{Val}$ the dissociation of the nascent $Val\text{-}tRNA^{Val}$.

Since it was demonstrated that the specificity of $tRNA^{Val}$ charging by yeast valyl-tRNA synthetase results from a kinetic process reducing strongly the rate of aminoacylation of noncognate tRNAs as compared to the rate of $tRNA^{Val}$ charging rather than from differences in the affinities of the various

tRNA species for the synthetase (Ebel et al., 1973), it can be expected that some of the partial steps detected in this study contribute to the specificity of the aminoacylation reaction. Further investigations are in progress to characterize these steps.

Acknowledgments

We are grateful to Professor Ebel in whose laboratory this work was done. We thank Dr. R. Giegé for stimulating discussions and R. Roads for reviewing the manuscript.

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