THE $^{32}PP_i$–ATP ISOTOPE-EXCHANGE REACTION CATALYZED BY THE YEAST VALYL-tRNA SYNTHETASE

Order of substrate binding and effect of tRNA

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1. Introduction

Beside the tRNA aminoacylation reaction, the aminoacyl-tRNA synthetases catalyze also the incorporation of $^{32}PP_i$ into ATP [1–5]. It is generally believed that this isotope-exchange occurs at the equilibrium of the amino acid activation step, which is the first step in the two-step mechanism often proposed for the tRNA aminoacylation reaction catalyzed by the synthetases [1–5]. This isotope-exchange reaction, and consequently the amino acid activation step, generally does not require the presence of tRNA, except in the case of the three enzymes specific for glutamine, glutamic acid and arginine [6–12].

This paper is a study of the $^{32}PP_i$–ATP exchange reaction catalyzed by yeast valyl-tRNA synthetase. Initial velocities, product and dead-end inhibition measurements are reported which indicate that ATP binds to the enzyme before valine, PP_i being released after the formation of the activated aminoacyl-adenylate : enzyme complex. Although valyl-tRNA synthetase does not require the presence of its cognate tRNA during the $^{32}PP_i$–ATP exchange, we have investigated its effect, as well as that of periodate-oxidized $tRNA^{Val}$ and of $tRNA^{Phe}$ on this exchange reaction. The experiments show that $tRNA^{Val}$, either native or periodate-oxidized, modifies the kinetic parameters of the isotope exchange, whereas the non-cognate $tRNA^{Phe}$ is without effect on this exchange.

2. Materials and methods

2.1. *Chemicals and reagents*

L-valine was purchased from Merck and L-[^{3}H]valine (spec. act. 30 Ci/mmol) from the Commissariat à l'Energie Atomique (Saclay); ATP, AMP, valylol and bovine serum albumin were from Sigma. AMPcPP (α,β methylene adenosine triphosphate) was from Aldrich, and $Na^{32}PP_i$ from Amersham. Unfractionated yeast tRNA was from Boehringer. All the other products were of the highest purity commercially available.

2.2. *Enzyme and tRNAs*

Yeast-valyl-tRNA synthetase (EC 6.1.1.9, $E^{280\,nm}_{1\,mg/ml/cm} = 1.77$) was purified as in [13]. The turnover measured by the aminoacylation at 37°C and pH 7.2 was of 8.0 s^{-1}. The major species of yeast $tRNA^{Val}$ (acceptance capacity 1500 pmol/A_{260}) was purified as in [13] and yeast $tRNA^{Phe}$ (acceptance capacity 1300 pmol/A_{260}) was obtained pure after counter current fractionation [14]; $tRNA^{Val}$ was oxidized in the presence of sodium periodate by the procedure in [15].

2.3. *$^{32}PP_i$–ATP isotope exchange reaction*

The incubation mixtures contained: 100 mM Tris–HCl (pH 7.5), ATP, $^{32}PP_i$ and L-valine at the indicated concentrations. The concentration of $MgCl_2$ was kept 5.0 mM in excess of the sum of PP_i and

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ATP (or PP_i, ATP and AMPcPP) concentrations. In some experiments $tRNA^{Val}$, $tRNA^{Val}_{ox}$ or $tRNA^{Phe}$ were added to the incubation mixtures. The valyl-tRNA synthetase, previously diluted in 50 mM Tris–HCl (pH 7.4) buffer containing 1 mg/ml bovine serum albumin and 5 mM 2-mercaptoethanol, was present in amounts allowing linear incorporation of $^{32}PP_i$ into ATP. After various incubation times at 28°C, 50 μl samples were precipitated into 0.5 ml of a suspension of acid-washed Norit (30% w/v) in 7.5% perchloric acid and 0.1 M Na PP_i. After filtration of the Norit on glass fiber Whatman filter discs GF/c the [^{32}P]ATP synthesized was determined by liquid scintillation counting. The kinetic results of $^{32}PP_i$–ATP exchange were expressed according to the terminology proposed [16].

2.4. *Aminoacylation of tRNA*

The incubation mixtures contained 100 mM Tris–HCl (pH 7.5), $MgCl_2$, ATP, L-[^{3}H]valine (75 000 cpm/nmol), $tRNA^{Val}$, AMP and $NaPP_i$ at the indicated concentrations. After various incubation times at 28°C, 50 μl aliquots were removed and the [^{3}H]val-tRNA synthesized determined as in [13].

3. Results

3.1. *Effects of ATP, valine and PP_i on the $^{32}PP_i$–ATP exchange reaction catalyzed by valyl-tRNA synthetase*

Kinetic evidence for a sequential pathway in the exchange reaction is obtained by varying ATP and L-valine at a fixed level of PP_i. The double reciprocal plots show a series of lines intersecting at the left of the vertical axis (fig.1A). Thus both substrates must add to the enzyme before a product is released.

When PP_i is varied at different fixed ATP or L-valine concentrations, the third substrate, respectively L-valine or ATP, being present at a constant concentration, the double reciprocal plots consist of parallel lines (fig.1B,C). Consequently ATP and L-valine on the one side, and PP_i on the other one, combine with enzyme forms which are irreversibly connected in the sequence of the various steps taking part in the $^{32}PP_i$–ATP isotope-exchange reaction.

3.2. *Effects of valylol and AMPcPP on the $^{32}PP_i$–ATP exchange reaction*

Valylol and AMPcPP which are structural analogues of valine and ATP were both found unable to stimulate the isotope-exchange reaction. Valylol acts as a competitive inhibitor with respect to valine (fig.2A). Thus valine and valylol bind to the same enzyme form. A similar competitive inhibition pattern is obtained when ATP is the varied substrate in the presence of changing fixed concentrations of AMPcPP, indicating that ATP and AMPcPP are also both able to bind to the same enzyme form (fig.3A). According to the definition [16] these analogues are dead-end inhibitors, and can therefore be used to determine the order of binding of ATP and valine to the enzyme. Valylol acts as an uncompetitive inhibitor respective to ATP (fig.2B), and AMPcPP as a non-competitive inhibitor respective to valine (fig.3B). Furthermore, both valylol and AMPcPP acts as uncompetitive inhibitors

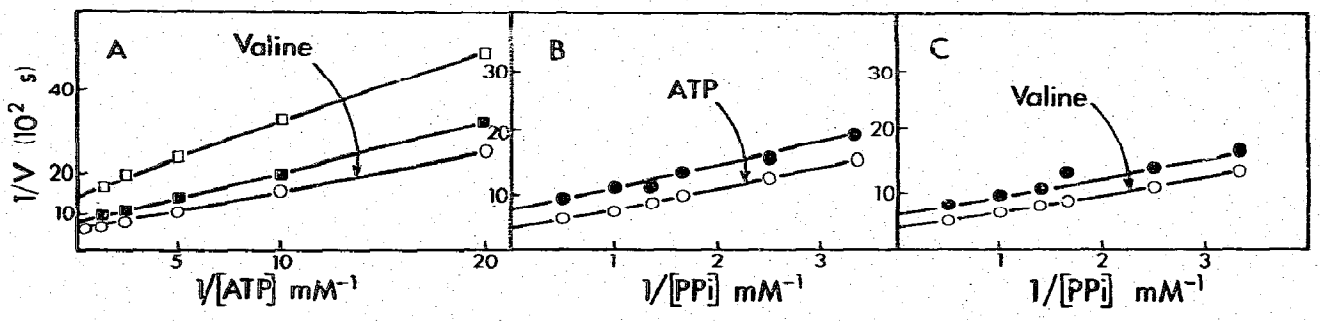


Fig.1. Plots of reciprocal exchange velocities against reciprocal concentrations of one substrate, in the presence of changing fixed concentrations of the second substrate and a constant concentration of the third substrate. (A) ATP variable, PP_i constant 1.0 mM and L-valine (□) 0.033 mM, (■) 0.1 mM, (○) 1.0 mM. (B) PP_i variable, L-valine constant 1.0 mM and ATP (●) 0.2 mM, (○) 1.0 mM. (C) PP_i variable, ATP constant 1.0 mM and L-valine (●) 0.1 mM, (○) 1.0 mM.

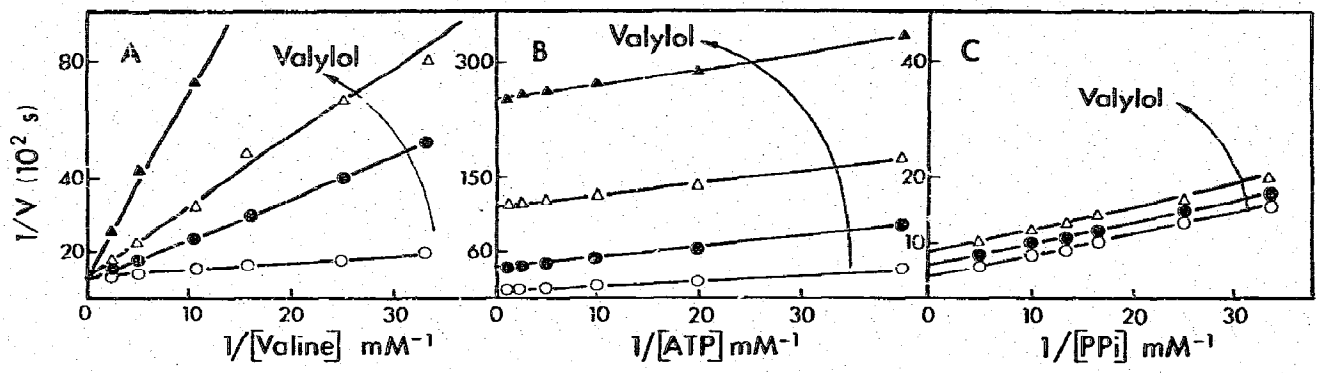


Fig.2. Inhibition of the valyl-tRNA synthetase catalyzed $^{32}PP_i$–ATP exchange by valylol. Double reciprocal plots with one substrate variable, at changing fixed concentrations of valylol and constant concentrations of the two other substrates. (A) L-valine variable, ATP 1.0 mM, PP_i 1.0 mM and L-valylol (○) 0 mM, (●) 0.06 mM, (△) 0.12 mM, (▲) 0.36 mM. (B) ATP variable, L-valine 0.2 mM, PP_i 1.0 mM and L-valylol (○) 0 mM, (●) 3.75 mM, (△) 12.5 mM, (▲) 25.0 mM. (C) PP_i variable, L-valine 0.5 mM, ATP 1.0 mM and L-valylol (○) 0 mM, (●) 0.4 mM, (△) 1.25 mM.

with respect to PP_i (fig.2C,3C). These kinetic patterns indicate that ATP binds before valine to the enzyme, and confirm that the enzyme forms binding ATP and valine are irreversibly connected to the enzyme form which binds PP_i.

The Michaelis constants derived from these studies are given in table 1.

3.3. *Effects of tRNAs of the $^{32}PP_i$–ATP exchange reaction*

Figure 4 shows that $tRNA^{Val}$, either charged or uncharged, can induce the same decrease of the reaction rate of the $^{32}PP_i$–ATP exchange reaction. In particular, no variations of the exchange reaction rate is detected when the amount of charged tRNA increases during the incubation time. In the presence of AMP, when the aminoacylation reaction is reversed, the tRNA effect is not modified.

The effect is also observed in the presence of $tRNA^{Val}_{ox}$, and is dependent upon the concentration of the tRNAs as shown in the inset of fig.4. The inhibition constants of the various forms of $tRNA^{Val}$ for the exchange, which are derived from the plots in the inset figure, and which can be assimilated to dis-

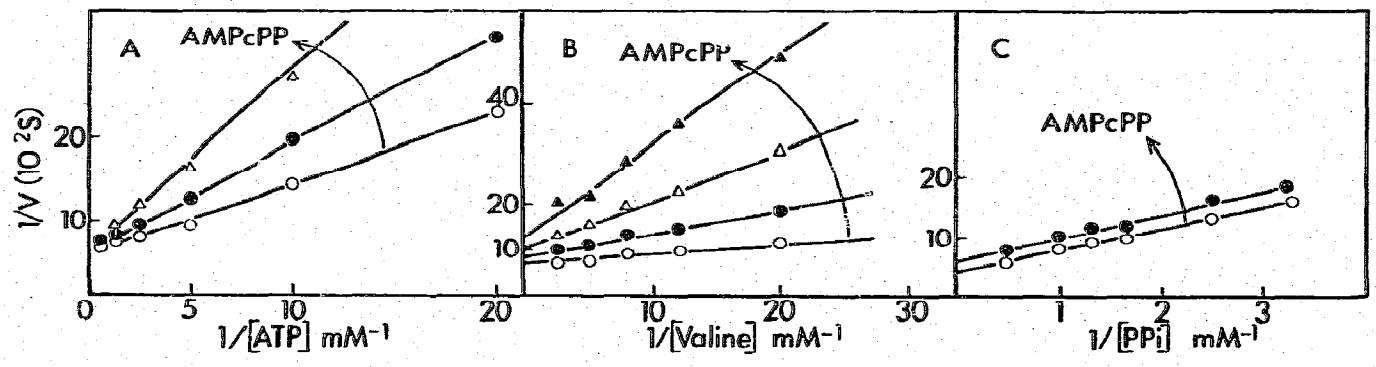


Fig.3. Inhibition of the valyl-tRNA synthetase catalysed $^{32}PP_i$–ATP exchange by AMPcPP. Double reciprocal plots with one substrate variable, at changing fixed concentrations of AMPcPP and constant concentrations of the two other substrates. (A) ATP variable, L-valine 1.0 mM, PP_i 1.0 mM, AMPcPP (○) 0 mM, (●) 2.5 mM, (△) 5.0 mM. (B) L-valine variable, ATP 0.5 mM, PP_i 1.0 mM, AMPcPP (○) 0 mM, (●) 1.25 mM, (△) 2.50 mM (▲) 5.0 mM. (C) PP_i variable, L-valine 0.5 mM. ATP 1.0 mM and AMPcPP (○) 0 mM, (●) 5.0 mM.

Table 1
Michaelis-Menten constants of the various ligands of yeast valyl-tRNA synthetase in the $^{32}PP_i$–ATP isotope-exchange reaction and inhibition constants of ligand analogues

Substrate or analogue	K_m (mM)		K_i (mM)
	– $tRNA^{Val}$	+ $tRNA^{Val}$	
L-valine	0.05[a]	0.20[a]	
L-valyl-ol			0.15[b]
ATP	0.20[a]	0.66[a]	
AMPcPP			5.0[b]
PP_i	1.00[a]	0.14[a]	

[a] The measurements are effected under the conditions in section 2 in the presence of limiting concentrations of the substrate tested, the concentrations of the two other ligands being constant: L-valine, 1.0 mM; ATP, 1.0 mM; PP_i, 1.0 mM
[b] The measurements are effected as in legends to fig.2A,3A.

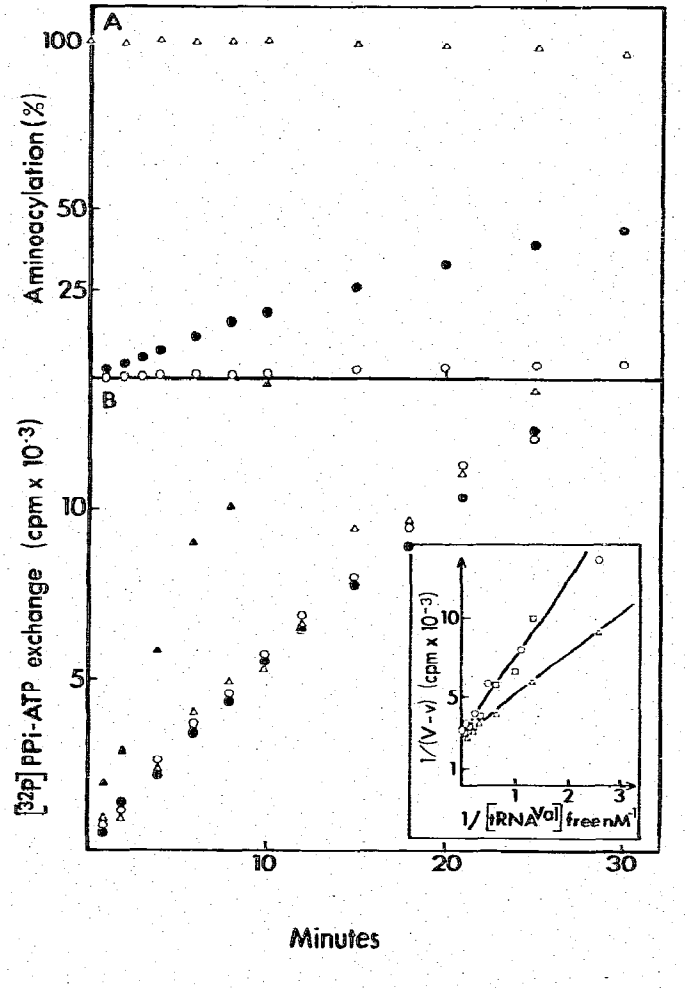


Fig.4. Correlation between the aminoacylation of $tRNA^{Val}$ and the $tRNA^{Val}$-induced inhibition of the $^{32}PP_i$–ATP exchange. (A) Aminoacylation of $tRNA^{Val}$. (B) $^{32}PP_i$–ATP exchange. Both incubation mixtures contained ATP 1.0 mM, L-valine 0.05 mM (^{3}H-labelled in A), PP_i 2.0 mM (^{32}P-labelled in B), $tRNA^{Val}$ 10 μM (△,○,●) or no $tRNA^{Val}$ (▲). In one experiment (△), $tRNA^{Val}$ was aminoacylated before starting the isotope exchange reaction which was initiated at $t = 0$ by addition of PP_i; in another experiment (○) 1.0 mM AMP was present. The inset of B shows the dependence of the inhibition of the $^{32}PP_i$–ATP exchange rate with respect to the concentration of the various $tRNA^{Val}$ forms ((○) uncharged; (△) charged; (□) periodate oxidized $tRNA^{Val}$). This dependence is presented according to the double reciprocal plot:

$$1/(V-v) = f\,(1/[tRNA^{Val}]free)$$

where V and v represent respectively the isotope-exchange rates in the absence of $tRNA^{Val}$ and in the presence of a given concentration of $tRNA^{Val}$; $tRNA^{Val}_{free}$ represents the concentration of $tRNA^{Val}$ not bound to the enzyme which was obtained by subtracting from the initially present $tRNA^{Val}$ concentration that of the Enzyme - $tRNA^{Val}$ complex. The concentration of this complex was determined assuming that the extent of inhibition is proportional to the saturation extent of the enzyme by $tRNA^{Val}$, the optimal inhibition corresponding to the saturation of the enzyme by tRNA. The concentrations of the reactants were: ATP 1.0 mM, $^{32}PP_i$ 2.0 mM, L-valine 0.05 mM, enzyme 1.75 nM and those of the various tRNAs varied between 0.4 nM and 1.75 μM. When val-$tRNA^{Val}$ was tested, the mixture containing $tRNA^{Val}$ was preincubated 30 min in the absence of PP_i; when uncharged $tRNA^{Val}$ was tested the incubation mixture contained 1.0 mM AMP.

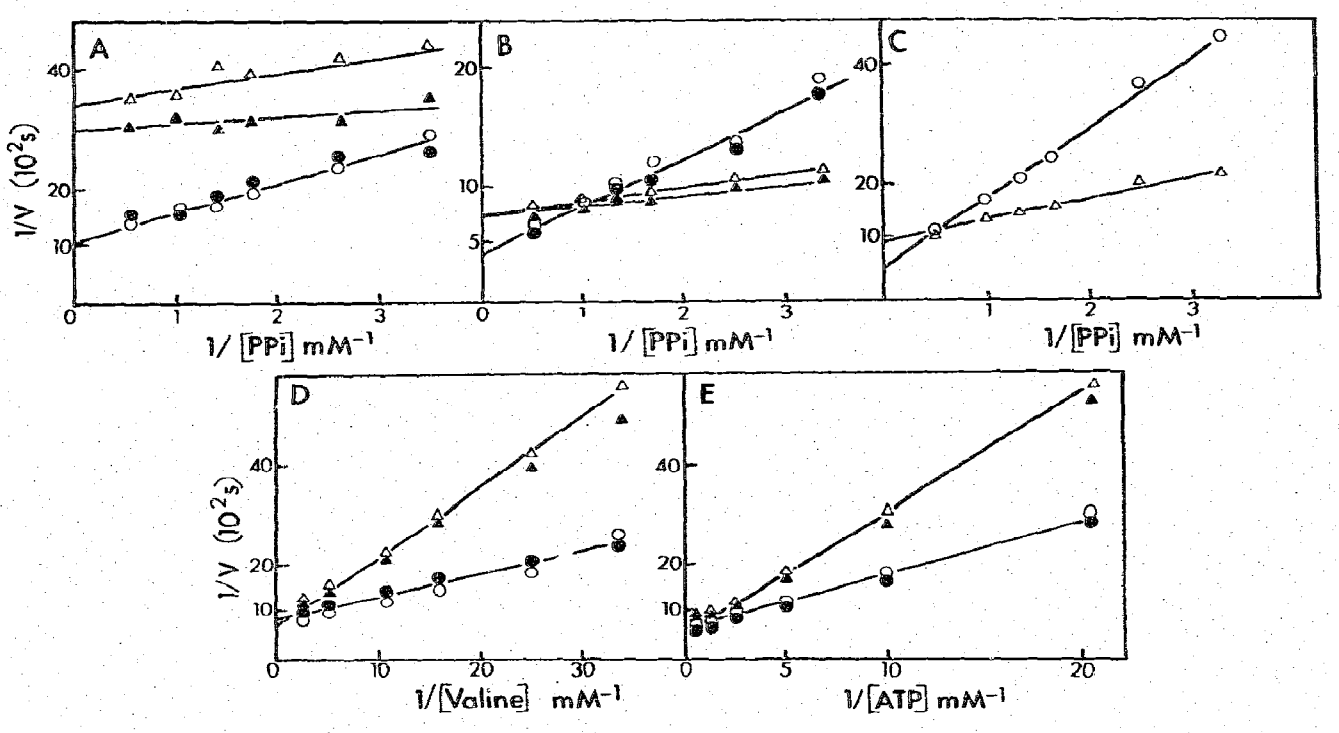


Fig.5. Effects of $tRNA^{Val}$, $tRNA^{Val}_{ox}$ and $tRNA^{Phe}$ on the $^{32}PP_i$–ATP exchange reaction. (A–C) double reciprocal plots with variable PP_i concentration and constant ATP and valine concentrations. (D,E) double reciprocal plots with variable valine or ATP concentrations and constant concentrations of the two other substrates. (A) ATP 1.0 mM, L-valine 0.05 mM. (B) ATP 1.0 mM, L-valine 1.0 mM. (C) ATP 10.0 mM, L-valine 5.0 mM. (D) PP_i 1.0 mM, ATP 1.0 mM. (E) PP_i 1.0 mM, L-valine 1.0 mM. The reactions were effected either without tRNA (○) or with 10 μM $tRNA^{Val}$ (△) or 10 μM $tRNA^{Val}_{ox}$ (▲) or 30 μM $tRNA^{Phe}$ (●).

sociation constants, are in the range of 1 nM.

The double reciprocal plots of fig.5 show the dependence of the tRNA effect with the concentration of the ligands involved in the isotope exchange. For various valine and ATP concentrations the apparent maximal velocity of the system is reduced and the K_m of PP_i for the synthetase is decreased by ~1 order of magnitude in the presence of a saturating $tRNA^{Val}$ concentration (fig.5A-C). The effect on the apparent maximal rate can however be strongly attenuated by an excess of ATP or valine (fig.5D,E). Indeed $tRNA^{Val}$ acts as a competitive inhibitor respective to ATP and valine, and increases the K_m values of these ligands for the enzyme. Finally at the same saturation extent of the enzyme by both ATP and valine (at concentrations corresponding respectively, to 5- and 20-times the K_m values determined in the presence of 1 mM PP_i) it was shown that the apparent maximal rates of the exchange measured in the absence or in the presence of $tRNA^{Val}$ become similar at very high PP_i concentration (results not shown).

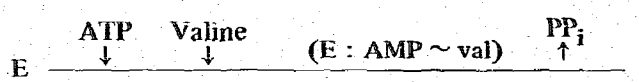
The comparison of the numerical values of the Michaelis constants of the three ligands involved in the $^{32}PP_i$–ATP exchange, obtained in the presence or in the absence of $tRNA^{Val}$ is given in table 1.

These $tRNA^{Val}$ effects are not modified by AMP (results not shown) and occur also with periodate-oxidized $tRNA^{Val}$ (fig.5). On the contrary, $tRNA^{Phe}$, which can strongly bind to valyl-tRNA synthetase, and which is easily misacylated by this synthetase [17] is without effect on the exchange (fig.5).

4. Discussion

The kinetic studies of the $^{32}PP_i$–ATP isotope-exchange reaction catalyzed by the yeast valyl-tRNA synthetase presented in this work allow us to propose the order of addition and of release of the substrates

and products which occur during the activation step of valine. The results can be represented by the following scheme:



which shows that ATP binds before valine to the enzyme. This sequential binding also occurs during the overall aminoacylation reaction catalyzed by this enzyme (D. K. et al., in preparation). Ordered additions of ATP and amino acid were also found for other synthetases, either by studying the isotope-exchange reaction as in the case of the *E. coli* leucine enzyme [18], or by studying the overall aminoacylation process, as in the case of the *E. coli* proline, the yeast leucine and the rat liver tryptophan enzymes [19–21]. This scheme, however seems not to be general for all aminoacyl-tRNA synthetases, since random associations of the ligands were described in other systems [22,23]. But it may be noticed that depending on the experimental conditions, alternative mechanisms have been proposed for a same enzyme, e.g., beef pancreas tryptophanyl-tRNA synthetase [24]; so that the existence of a similar behaviour of all synthetases cannot be excluded.

The yeast valyl-tRNA synthetase belongs to the enzymes which catalyze the $^{32}PP_i$–ATP isotope-exchange in the absence of tRNA [13]. Nevertheless, in the present work we present evidence that the cognate $tRNA^{Val}$, similarly to other tRNAs in different systems [25–31], influences this exchange reaction. The effect we observed here is, however, not related to the tRNA effect occurring in the glutamic acid, glutamine and arginine systems, where the presence of the tRNA is obligatory for promoting the isotope-exchange, probably by allowing the binding of ATP and/or amino acid to the enzyme [6–12]. Furthermore, our experiments demonstrate that the $tRNA^{Val}$-induced effect is not linked to the coupling of the transfer step of the activated amino acid to the tRNA with the isotope-exchange reaction. Indeed such a coupling of both reactions using the same enzyme : adenylate intermediate would result in a competition between $tRNA^{Val}$ and PP_i for this intermediate. Thus, the presence of accepting $tRNA^{Val}$ should apparently decrease the affinity of the enzyme for PP_i and reduce the maximal rate of the isotope-exchange; in addition, this $tRNA^{Val}$-effect should be reversed by an excess of PP_i. Our results show opposite behaviour: indeed, the affinity of the enzyme for PP_i increases in the presence of $tRNA^{Val}$ as shown by a 10-fold decrease of the K_m (see table 1) and the $tRNA^{Val}$-induced decrease of the apparent maximal rate of the isotope-exchange is not reversed in the presence of an excess of PP_i. Furthermore, the presence of AMP, by reversing the transfer step, should reduce the $tRNA^{Val}$ effect: we did not observe such an effect. Finally, if $tRNA^{Val}$ would significantly compete with PP_i, the presence of $tRNA^{Val}_{ox}$ should not affect this isotope-exchange: our data show that $tRNA^{Val}_{ox}$ can efficiently replace native $tRNA^{Val}$. Another explanation of this phenomenon could be that $tRNA^{Val}$ induces the hydrolysis of adenylate, as shown for $tRNA^{Phe}$ in the presence of yeast phenylalanyl-tRNA synthetase [32], so that the catalytically available concentration of adenylate would be lower in the presence of tRNA than in its absence. Such a mechanism would consume ATP. We verified, in the presence of $tRNA^{Val}_{ox}$, which cannot be aminoacylated, that no significant amount of ATP is consumed during the isotope-exchange.

One may now wonder about the mechanism by which $tRNA^{Val}$ influences the exchange reaction. We propose that it is related to the specific interaction of the tRNA with the enzyme. Let us recall that the rate of the exchange reaction depends upon the rates of various partial steps capable of being rate-limiting: those of the two catalytic steps establishing the equilibrium, and those of the exchange steps between free and enzyme-bound PP_i and ATP. The decrease of the apparent maximal rate of the isotope-exchange and the decrease of the app. K_m of PP_i suggest a priori a slow dissociation of PP_i from the (E : AMP ~ Val, PP_i, $tRNA^{Val}$) complex. However as an excess of ATP or valine is able to reverse the $tRNA^{Val}$-induced decrease of the apparent maximal rate, it appears that even if the affinity of PP_i for the (E : AMP ~ Val, $tRNA^{Val}$) complex is increased, the rate of exchange between the free and the enzyme-bound PP_i is not rate-limiting in the $^{32}PP_i$–ATP exchange effected in the presence of $tRNA^{Val}$. It seems thus, that the decrease of the rate we observed is rather linked to the formation of inactive (Enzyme, $tRNA^{Val}$, PP_i) complexes able to

be reversed either by an excess of ATP or valine. Our results therefore suggest:

(i) That PP_i can combine with two different enzyme forms, E : AMP ~ Val and free enzyme;

(ii) That $tRNA^{Val}$ increases significantly the affinity of PP_i for these two enzyme forms.

Kinetic studies of the overall aminoacylation reaction are in progress to elucidate this mechanism in more detail.

We suggest that the particular kinetic behaviour of the exchange reaction effected in the presence of $tRNA^{Val}$ is the consequence of a conformational change of the synthetase induced by the nucleic acid. This effect is specific since the non-cognate $tRNA^{Phe}$ although it can be aminoacylated by yeast valyl-tRNA synthetase [17] did not provoke it. Conformational changes of synthetases induced by the cognate tRNA have already been described [33–35]. In particular, in the case of the yeast valine system, a contraction of the enzyme upon $tRNA^{Val}$-binding was detected by neutron small angle scattering methods [35]. The question however remains open whether the kinetic effects described in this work can be directly correlated to this contraction or if they result from more discrete events occurring during the interaction of the tRNA with the synthetase.

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