Valyl-tRNA Synthetase from Baker's Yeast. Ligand Binding Properties and Stability of the Enzyme-Bound Adenylate†

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ABSTRACT: The stoichiometry of the binding sites of the various ligands (substrates and end products) on the monomeric yeast valyl-tRNA synthetase and their coupling have been investigated by various approaches. The synthetase possesses one adenylation site and binds one valine and one $tRNA^{Val}$. However, whereas also one ATP (or AMP and PP_i) binds in the presence of magnesium, two ATP or PP_i (but only one AMP) bind in its absence. The synthetase binds other nucleoside triphosphates such as GTP or CTP. No interrelation between the binding sites of valine and $tRNA^{Val}$ has been detected; however, in the absence of magnesium (when no adenylate is generated) the valine binding site and both PP_i (or nucleoside triphosphates) binding sites were found coupled. On the other hand, no simple coupling was found between the valine and the AMP sites: AMP did not promote the binding of valine, whereas valine promoted that of AMP and generated in the presence of magnesium a second site for AMP. Since $tRNA^{Val}$ always suppressed the second binding site for ATP, AMP, and PP_i, it appears that these small ligands bind to a tRNA interacting site. It is proposed that this site corresponds to the binding site of the adenosine end of $tRNA^{Val}$ and that valine and either the whole tRNA or magnesium promote this binding. Evidence is brought indicating that valyl-tRNA synthetase possesses only one catalytic center and does not dimerize during the catalytic process. The properties of the enzyme-bound adenylate have been investigated. The complex exhibited an unusually high stability. However, its storage at 4 °C was accompanied by a slow dissociation; the final equilibrium was established after ~8 days. The freshly prepared complex was destabilized by the small substrates and nonaccepting $tRNA^{Val}$ (periodate oxidized or charged) especially in the absence of PP_i. The study of the stability of the adenylate complex in the presence of various periodate-oxidized yeast tRNAs and in the presence of periodate-oxidized $tRNA^{Val}$ at various pHs showed a correlation between the ability of a tRNA to be aminoacylated by the synthetase and its ability to labilize after periodate oxidation the enzyme-bound adenylate. It is proposed that the association of either ATP or the CCA end of a nonaccepting tRNA with the enzyme·adenylate complex promotes the ejection of Val~AMP from its specific binding site and that the labilization of the complex by the CCA end of the tRNA could be necessary for its aminoacylation. Finally, the specificity of the amino acid activation reaction catalyzed by this synthetase has been investigated.

It is now well established that all aminoacyl-tRNA[1] synthetases catalyze the tRNA charging in a stepwise mechanism involving the adenylate as an obligatory intermediate [see, for example, Eldred & Schimmel (1972), Fersht & Jakes (1975), Fersht & Kaethner (1976), Kim et al. (1977) Lui et al. (1978), Fersht et al. (1978), Godeau (1980), Kern & Lapointe (1980a,b)]. However, when the mechanistic behavior of these enzymes is deeply investigated, large discrepancies appear. This is, for example, the case for the number of binding sites of the ligands, for the order of binding of these ligands, and for the rate-determining step in the catalytic process [see the general reviews by Söll & Schimmel (1974), Kisselev & Favorova (1974), Kalousek & Konigsberg (1975), and Schimmel & Söll (1979)]. These differences could be related to the structural heterogeneity of these enzymes [i.e., small monomers (M_r 56 000–75 000) without or with limited sequence duplications, large monomers (M_r ~120 000) with significant sequence duplications, dimers of the α_2 type with small (M_r 2 × 50 000) or large (M_r 2 × 80 000) subunits, and tetramers of the $\alpha_2\beta_2$ type (see the general reviews)]. If so, the synthetases belonging to a particular structural class should have similar functional properties. This is, for example, true for the small monomeric and for the dimeric and tetrameric synthetases as far as the number of binding sites of ligands is concerned; indeed, the synthetases of these groups bind respectively one, two, and two of each substrate and act in their native form [see, for example, Bruton & Cox (1979) and Kern & Lapointe (1979a) concerning the small monomers and the general reviews for the dimeric and tetrameric synthetases]. Surprisingly, however, there is contradictory information concerning the large monomers of molecular weight ~120 000. Indeed it was shown that some enzymes of this group possess two binding sites for the amino acid (Fersht, 1975; Fersht & Kaethner, 1976; Moe & Piszkiewicz, 1979), tRNA (Von der Haar & Cramer, 1978), or even adenylate (Fersht, 1975) whereas some others would possess only one binding site for these ligands (Hustedt & Kula, 1977a,b). The existence of two catalytic sites would indicate that the synthetases of this group act as dimers in a manner similar to the α_2 dimeric and $\alpha_2\beta_2$ tetrameric synthetases and would give a functional interpretation of their large sequence duplications. Alternatively, it has also been postulated that these enzymes are true monomers which could dimerize during the catalytic process (Österberg et al., 1975).

In the present work we have undertaken a study of the ligand binding properties of valyl-tRNA synthetase from

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[1] Abbreviations used: tRNA, transfer ribonucleic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Na_2EDTA, disodium (ethylenedinitrilo)tetraacetate; DTE, dithioerythritol; PP_i, pyrophosphate; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; CTP, cytidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; PPase, inorganic pyrophosphatase; ValRS, valyl-tRNA synthetase; aa~AMP, aminoacyl-adenylate; $tRNA_{ox}^{aa_1}$, periodate-oxidized tRNA for aa_1; aa-tRNA, charged tRNA; Tris, tris(hydroxymethyl)aminomethane.

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baker's yeast, a representative enzyme of the group of large monomeric synthetases. Several structural and kinetic properties of this synthetase have already been reported (Rymo et al., 1972; Kern et al., 1975, 1980; Bruton, 1975; Giegé et al., 1977; Lagerkvist et al., 1977; Fersht & Dingwall, 1979; Kern & Giegé, 1979), but the ligand binding properties of this enzyme as well as those of other synthetases from the same structural group have never been exhaustively studied. In this work we will try to answer to several questions. What is the actual number of binding sites for the ligands on this synthetase? Are there interrelations between these sites, and if so, what is the nature of these interrelations? Does the native monomeric synthetase dimerize during the catalytic process? This last aspect has been investigated by sucrose gradient centrifugation of the enzyme under catalytic conditions, and we bring evidence that the synthetase is fully active in its monomeric form. However, the existence of two similar domains (Bruton, 1975; Kern et al., 1975) could indicate that this synthetase is in fact a pseudodimer possessing two catalytic sites. Its ligand binding properties were thus studied in a large range of concentrations in order to detect even strong anticooperative binding sites. They indicate that the synthetase possesses one adenylation site in which the binding site of ATP and valine are coupled and one binding site for tRNA. In addition to the adenylate site, ATP can also bind to a tRNA interacting site; since valine and magnesium modulate the interaction of either the AMP or the PP_i part of ATP in this site, it is postulated that it corresponds to the binding site of the adenosine end of the tRNA. The results are interpreted in the context of the structure–function relationship of the enzyme. The specificity of the adenylation reaction has been investigated, and we report some peculiar properties of correct and wrong enzyme-bound adenylates.

Experimental Procedures

General. L-[^{14}C]Valine, L-[^{14}C]threonine, [^{14}C]ATP,[1] [^{14}C]AMP,[1] and [^{14}C]CTP[1] (250, 200, 300, 460, and 470 mCi/mmol, respectively) were from the Commissariat à l'Energie Atomique, [^{32}P]PP_i[1] was from Amersham, France, and [α-^{32}P]ATP,[1] [γ-^{32}P]ATP,[1] and [γ-^{32}P]GTP[1] were from New England Nuclear. L-Amino acids were from Merck. Their purity was checked on a Durrum D500 analyzer. They did not contain a measurable contamination of valine ($\ll 1/10\,000$). ATP, AMP, CTP, GTP, Hepes,[1] Mes,[1] DTE,[1] and inorganic pyrophosphatase (1 unit catalyzed the formation of 1 μmol of inorganic phosphate per min at 25 °C and pH 7.4) were from Sigma. Unfractionated yeast tRNA was from Boehringer; Sephadex G-25, G-75, and G-200 (Superfine) were from Pharmacia; nitrocellulose disks (pore size, 0.22 μm) were from Millipore and glass fiber disks (GF/c) from Whatman. Omnifluor scintillator was from New England Nuclear.

Enzyme and tRNAs. Valyl-tRNA synthetase (EC 6.1.1.9) from yeast was prepared as described previously (Kern et al., 1975). The turnover, measured in the presence of the standard aminoacylation mixture 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.

Pure brewer's yeast $tRNA^{Val}$ (major species), $tRNA^{Arg}$, $tRNA^{Asp}$, $tRNA^{Gly}$, $tRNA^{Leu}$, $tRNA_f^{Met}$, and $tRNA^{Phe}$ (acceptance capacity 1300 to 1600 pmol/A_{260nm}) were obtained after counter-current fractionation (Dirheimer & Ebel, 1967), followed by conventional chromatographic techniques. Periodate oxidation of the tRNAs was done in the presence of 2 mM sodium periodate (Kern et al., 1972).

Preparation of Enzyme·Adenylate Complexes. The enzyme·[^{14}C]Val~AMP and enzyme·[^{14}C]Thr~AMP complexes were prepared by incubating valyl-tRNA synthetase (25 μM) in the presence of 50 mM NaHepes buffer, pH 7.2, 50 μM [^{14}C]valine (27.2 cpm/pmol) or 200 μM [^{14}C]threonine (31 cpm/pmol), 1 mM ATP, 2 mM $MgCl_2$, and 10 units of inorganic pyrophosphatase per mL for 5 min at 25 °C. The complexes were then separated from excess of free ligands by filtration on a Sephadex G-75 column (0.5 × 30 cm) equilibrated with 100 mM Tris-HCl buffer, pH 7.4, or 50 mM NaHepes buffer, pH 7.0, containing 5 mM β-mercaptoethanol and 0.2 mM DTE.

Titration of Active Sites of Valyl-tRNA Synthetase. (*1*) *Measurements of the Cleavage of* [γ-^{32}P]*ATP.* The compositions of the reaction mixtures were as described under Results. After various incubation times at the indicated temperature, 20- or 50-μL aliquots were transferred in 180 μL of a suspension of acid-washed Norit (1%) in 7% perchloric acid and 0.1 M PP_i. After centrifugation the [^{32}P]PP_i was determined by counting a 100-μL aliquot of the supernatant in 10 mL of Bray's solution (Bray, 1960). The [γ-^{32}P]ATP adsorbed on the Norit was determined after filtration on glass fiber disks (GF/c) as described previously (Kern et al., 1975).

(*2*) *Nitrocellulose Disk Filtration of the Enzyme·Adenylate Complex.* The synthetase was diluted to a final concentration of 3.5 μM in 100 mM NaHepes buffer, pH 7.0, containing 5 mM β-mercaptoethanol, either 1 mM or 10 μM ATP [either α-^{32}P-labeled (50 cpm/pmol) or not labeled], either 1 mM or 10 μM L-valine [either not labeled or ^{14}C-labeled (21 cpm/pmol)], and 2 mM $MgCl_2$. After various incubation times at 0 or 25 °C, 50- or 100-μL aliquots were filtered through nitrocellulose disks which were then washed 3 times with 2 mL of a solution of 100 mM NaHepes buffer, pH 7.0, containing 5 mM β-mercaptoethanol. After drying, the labeled material retained on the disks was counted in the presence of 2 mL of Omnifluor scintillator.

tRNA Valylation Reactions. The standard aminoacylation mixture contained 50 mM Tris-HCl, pH 7.4, 30 mM KCl, 5 mM ATP, 12 mM $MgCl_2$, 0.1 mM L-[^{14}C]valine (50 μCi/μmol), either 5 mg of unfractionated tRNA/mL or 1–10 μM $tRNA^{Val}$ (or another specific tRNA), and an adequate amount of valyl-tRNA synthetase (1–10 nM when $tRNA^{Val}$ was charged, 1–10 μM when another tRNA was charged). After various incubation times at 37 °C, the synthesized aminoacyl-tRNAs were determined as described previously (Kern et al., 1975).

Measurements of the Stability of the Isolated Enzyme·Adenylate Complexes under Various Conditions. The enzyme [^{14}C]Val~AMP or enzyme·[^{14}C]Thr~AMP complexes were incubated in 50 mM NaHepes buffer, pH 7.0, containing 5 mM β-mercaptoethanol and substrates as indicated in each case. After various incubation times at the indicated temperature, 50- or 100-μL aliquots were filtered through nitrocellulose disks; after being washed 3 times with 2 mL of a solution of 50 mM NaHepes buffer, pH 7.0, containing 5 mM β-mercaptoethanol and dried, the labeled material retained on the disks was counted in the presence of 2 mL of Omnifluor scintillator.

Equilibrium Dialysis. The apparatus containing eight cells was constructed according to Furlong et al. (1972). One chamber of each cell contained valyl-tRNA synthetase and when present one or several ligands (not labeled) at a saturating concentration in 50 mM Tris-HCl buffer, pH 7.4, 10% glycerol, and 5 mM β-mercaptoethanol. The other chamber contained the labeled ligand whose binding with the synthetase

was investigated. Each chamber was filled with a 55-μL solution of reactants whose concentrations are indicated in each case. The two chambers were separated with a prewashed cellulose membrane cut from dialysis tubes (from Union Carbide Corp.). Equilibration of valine and AMP was reached after 6 h. PP_i and ATP dialyzed slowly through these membranes; therefore, more permeable IRIS 3069 membranes (from the Societé des Usines Chimiques Rhône Poulenc) were used. Equilibration of ATP and PP_i was then reached within 5 h. After equilibration a 40-μL aliquot from each compartment was diluted in 10 mL of Bray's solution and counted in a liquid scintillation counter. Routine verifications at the end of dialysis showed that 80–100% of the enzyme activity was recovered and that no hydrolysis of ATP occurred during the experiments. Data were treated according to the Scatchard representation

$$\nu = [L]_b/[E]_t = f([L]_b/[L]_f) \quad (1)$$

where $[L]_b$, $[L]_f$, and $[E]_t$ are respectively the concentrations of bound and free ligand at the equilibrium and of the total enzyme. $[L]_f$ is determined directly from the radioactivity in the compartment deprived of enzyme, and $[L]_b$ is determined from the difference of radioactivity between the two compartments containing or not the enzyme. In the case of one binding site the dissociation constant K_D is given by the slope of the Scatchard plot. In the case of two binding sites, the Scatchard plot will be biphasic, and assuming that the two sites are independent, their dissociation constants K_1 and K_2 will be related with ν and $[L]_f$ according to

$$\nu = [2[L]_f/K_1 + 2[L]_f^2/(K_1K_2)]/[1 + 2[L]_f/K_1 + [L]_f^2/(K_1K_2)] \quad (2)$$

Equilibrium Gel Filtration of the Small Ligands. Sephadex G-50 (1 mL) was packed in a 1-mL tuberculin syringe and equilibrated with 50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, the labeled ligand whose binding with the enzyme was investigated, and, when present, other ligands (not labeled) at a saturating concentration. A sample of 100 μL containing the synthetase in the equilibrium buffer was filtered through the column. The flow rate was 1 drop/min; individual drops of eluant were collected on a plastic sheet, their volume was measured with a Hamilton syringe, and their radioactivity was determined by liquid scintillation after dilution in 10 mL of Bray's solution. All measurements were performed after saturation of the column with the enzyme. We verified that under these conditions 100% of the synthetase activity initially present in the sample was recovered after filtration. The dissociation constants were calculated according to $K_D = [E]_f[L]_f/[L]_b$ in the case of one binding site and according to eq 2 in the case of two independent binding sites. $[L]_f$ corresponds to the concentration of the ligand in the equilibrium buffer. $[E]_f$, the free enzyme concentration, and $[L]_b$ cannot be determined directly. They are obtained as a ratio $x/y = [E]_f/[L]_b$ where x and y are picomoles of E_f and L_b, respectively; y was measured directly from the radioactivity peak in the elution pattern (which corresponds to the radioactivity of $L_f + L_b$) or from the trough (which corresponds to the radioactivity of $L_f - L_b$); x is given by $E_t - y$, where E_t is the total amount of enzyme in the 100-μL sample deposited on the column.

Sucrose Gradient Centrifugations. The methodology used was that described by Martin & Ames (1961). The 5–20% sucrose gradients (10 mL) contained 50 mM NaMes, pH 5.8, 0.1 mM DTE, 5 mM β-mercaptoethanol, 0.1 mM diisopropyl fluorophosphate, 10 mM $MgCl_2$, and, when present, the substrates at following concentrations: 2 mM ATP, 1 mM L-valine, and 20 mg/mL unfractionated yeast tRNA. Samples (100 μL) of the above-described buffer containing either 500 pmol of valyl-tRNA synthetase and, when present, the various substrates at the indicated concentrations or 1500 pmol of purified $tRNA_2^{Val}$ were loaded on the top of the gradients. After centrifugation at 40 000 rpm at 4 °C for 18 h in a SW 41 Beckman rotor, 235-μL fractions were collected from the bottom of the gradients and assayed for synthetase activity in the standard tRNA aminoacylation mixture. Catalase was used to standardize the gradients and was detected according to Chance & Maehly (1955).

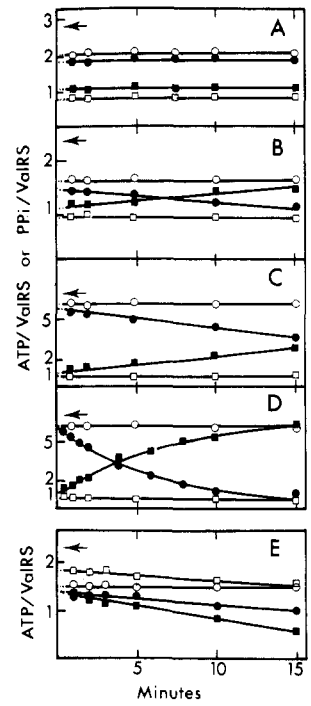

FIGURE 1: Titration of the adenylate sites of valyl-tRNA synthetase by the active-site titration approach under various experimental conditions: (A–D) in the absence of tRNA; (E) in the presence of tRNAs. The incubation mixtures contained 100 mM Tris-HCl, pH 7.4, 1 mM L-valine, 3.74 μM valyl-tRNA synthetase, $MgCl_2$ and [γ-^{32}P]ATP (50 cpm/pmol) at the indicated concentrations, and either no (○, □, △, ▽) or 2 units of inorganic pyrophosphatase/mL (●, ■, ▲, ▼). (A and B) 2 mM $MgCl_2$ and 10 μM ATP, incubations at 0 (A) or 25 °C (B). (C and D) 10 mM $MgCl_2$ and 28 μM ATP, incubations at 0 (C) or 25 °C (D). (E) 2 mM $MgCl_2$, 9 μM ATP, and either 6 μM $tRNA_{ox}^{Val}$ (△, ▲) or 6 μM $tRNA_{ox}^{Phe}$ (▽, ▼), incubations at 25 °C. The remaining [γ-^{32}P]ATP (○, ●, △, ▲, ▽, ▼) or the liberated [^{32}P]PP_i (□, ■) was determined. In control experiments, conducted in the absence of valine, no radioactive PP_i was released and no ATP was degraded, the pyrophosphatase being present or not (results not shown). The arrows indicate the ATP level at the beginning of the experiments at $t = 0$.

Results

Binding of the Ligands to Valyl-tRNA Synthetase: Stoichiometry and Interrelation of the Sites. (1) Adenylate. By use of the active-site titration approach (Fersht et al., 1975), it was found that at 0 °C, at pH 7.4, and in the presence of 2 mM magnesium and 1 mM valine, the ATP cleaved (and PP_i released) was with the synthetase in a 0.85 to 1 and 1 to 1 stoichiometric ratio in the absence and in the presence of inorganic pyrophosphatase, respectively. In both cases, the ATP consumption and PP_i release rapidly reached a plateau, indicating that under these conditions a stable enzyme-bound adenylate was synthesized (Figure 1A). At 25 °C however, the kinetics of ATP consumption were different whether pyrophosphatase was present or not. In its absence a stable plateau was reached after a rapid consumption of 0.85 mol

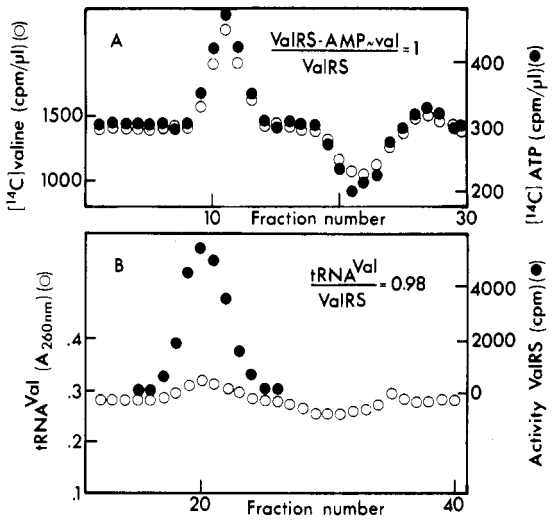

FIGURE 2: Stoichiometry of the binding sites of adenylate (A) and $tRNA^{Val}$ (B) on valyl-tRNA synthetase studied by equilibrium gel filtration. (A) The Sephadex G-50 column was equilibrated at 25 °C with 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, either 1 mM ATP and 52 μM L-[^{14}C]valine (27.3 cpm/pmol) (○) or 1 mM L-valine and 48 μM [^{14}C]ATP (6.3 cpm/pmol) (●), and 2 units of pyrophosphatase/mL. The 100-μL sample contained in the equilibrium buffer 3120 pmol of valyl-tRNA synthetase. The eluted fractions had a volume of ~50 μL. (B) The Sephadex G-200 column (1 × 38 cm) was equilibrated at 4 °C with 10 mM NaMes, pH 6.2, 5 mM β-mercaptoethanol, 0.1 mM DTE, 10 mM $MgCl_2$, and 2.2 μM $tRNA^{Val}$ (1 A_{260nm} = 35 μg). A 300-μL sample containing 2 μM valyl-tRNA synthetase in the equilibrium buffer was loaded on the column, and 550-μL fractions were collected. The A_{260nm} (○) was determined in a 0.2-cm path length cell, and the synthetase activity (●) was determined after a dilution of 1250-fold in a complete tRNA charging mixture. In both experiments the stoichiometries of the complexes, as determined from peak and from trough are indicated.

ATP/mol of synthetase, whereas in its presence the kinetics of ATP consumption were biphasic. After a first fast phase, during which one ATP was cleaved (and one PP_i released) per synthetase, the ATP was further cleaved with a much slower rate until complete consumption (Figure 1B). The rate of this second slow phase was independent of the concentration of pyrophosphatase (from 2 to 10 units/mL) but increased with the concentration of the synthetase (not shown). Consequently, the overconsumption of ATP was mediated by the synthetase. Finally, the increase of the magnesium concentration from 2 to 10 mM increased the rate of ATP consumption in the second phase at 0 and at 25 °C when pyrophosphatase was absent (Figure 1C,D). These results suggest that valyl-tRNA synthetase possesses one catalytic site for adenylate and show that in a complete reaction mixture at 25 °C the presence of pyrophosphatase destabilizes the enzyme-bound adenylate. This could a priori be related to a stabilizing effect of the ValRS·Val~AMP complex induced by PP_i. A 1 to 1 stoichiometric enzyme·adenylate complex was also isolated by gel filtration under equilibrium conditions by using either labeled ATP or valine (Figure 2A). However, the former two experimental methods do not allow titration of this site in the presence of high concentrations of both ATP and valine, so that the existence of a second adenylate site on the synthetase (which would either synthesize the adenylate slower or catalyze its pyrophosphorolysis faster than the first one) cannot be excluded. To test this possibility, we titrated the adenylate sites by nitrocellulose disk filtration, a method which allows the titration in the presence of high ATP and valine concentrations. This was possible because neither valine nor ATP remains associated to the synthetase when filtered through the nitrocellulose disks, whereas Val~AMP does. In the presence

Table I: Stoichiometry of Valyl-tRNA Synthetase Bound Val~AMP As Determined by Nitrocellulose Disk Filtration[a]

	extent of saturation of enzyme by			
	[^{14}C]Val~AMP[b]		Val~[^{32}P]AMP[c]	
conditions	−PPase	+PPase	−PPase	+PPase
1	0.80	1.00	0.84	1.02
2	0.98	0.99	0.98	1.00
3	0.80	0.98	0.87	0.97
4	0.86	0.87	0.85	0.82

[a] The incubation mixtures contained 100 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 3.5 μM valyl-tRNA synthetase, either 10 μM L-valine and 10 μM ATP (conditions 1 and 3) or 1 mM L-valine and 1 mM ATP (conditions 2 and 4), and, when present, 2 units of pyrophosphatase/mL. The reactions were conducted at 0 °C (conditions 1 and 2) or at 25 °C (conditions 3 and 4), and the measurements were performed after 1–30 min of incubation. [b] Incubation mixtures contained L-[^{14}C]valine. [c] Incubation mixtures contained [α-^{32}P]ATP.

of 1 mM ATP and valine, one of these ligands being labeled, the enzyme·adenylate complex retained on the disks was in a 1 to 1 stoichiometric ratio at 0 °C, either in the absence or in the presence of pyrophosphatase (Table I). The lower ratio (1 to 0.85) found at 25 °C (Table I) was due to a destabilization of the complex induced by the high substrate concentrations (see below). In the presence of low ligand concentrations results similar to those obtained with the former methods were obtained (Table I), establishing thus the accuracy of the nitrocellulose disks filtration technique.

In the presence of $tRNA^{Val}_{ox}$ the stoichiometries of ATP cleaved per synthetase were 0.5 to 1 and 1 to 1 in the absence and in the presence of inorganic pyrophosphatase, respectively (Figure 1E). Similar stoichiometries were obtained in the presence of Val-$tRNA^{Val}$ (not shown). This indicates that the cognate tRNA, either periodate oxidized or aminoacylated, displaces the equilibrium of the adenylation reaction toward the free reactants probably by increasing the rate of pyrophosphorolysis of the adenylate. $tRNA^{Phe}_{ox}$ does not affect the stoichiometry of the adenylate in the absence of pyrophosphatase (Figure 1E) and thus does not affect this equilibrium.

(*2*) *$tRNA^{Val}$ and Val-$tRNA^{Val}$.* When the synthetase was filtered through a Sephadex G-200 column equilibrated with $tRNA^{Val}$, a 1 to 1 stoichiometric enzyme·$tRNA^{Val}$ complex was eluted (Figure 2B). A similar stoichiometry was also determined by nitrocellulose disk filtration of the enzyme·Val-$tRNA^{Val}$ and enzyme·$tRNA^{Val}$ (respectively labeled on the valine moiety and on the adenosine end) complexes (not shown). Dissociation constant (K_D) values of ~10^{-8} M were determined for both complexes at pH 6.8, at 4 °C, and in the absence of magnesium (Kern et al., 1980). Neither the presence of magnesium nor that of ATP and valine (when adenylate was generated) affected significantly the K_D of Val-$tRNA^{Val}$ for the synthetase (Bonnet & Ebel, 1975).

(*3*) *Valine.* (See Table II and Figure 3.) In the concentration range from 10 μM to 2 mM (43 μM enzyme present) only one binding site for valine could be detected on the synthetase by equilibrium dialysis (K_D = 67 μM at 4 °C and pH 7.5). The presence of 10 mM magnesium decreased slightly this K_D (45 μM), but the presence of $tRNA^{Val}$ either in the absence or in the presence of magnesium did not affect it (Figure 3A). Under conditions where no adenylate could be synthesized (no magnesium and presence of Na_2EDTA[1]), ATP at a saturating concentration decreased strongly the K_D (67 and 9 μM in the absence and in the presence of ATP; Figure 3A). When ATP and magnesium were present to-

Table II: Binding of Small Ligands to Valyl-tRNA Synthetase: Stoichiometry of Binding Sites (n) and Dissociation Constants (K_D)[a]

Val		ATP		AMP		PP_i			
n	K_D (μM)	n	K_D (μM)	n	K_D (μM)	n	K_D (μM)	$MgCl_2$	$tRNA^{Val}$
1[b,c]	67[b]	–		–		–		–	–
1[b,c]	45[b]	–		–		–		+	–
1[b,c]	9[b]	+		–		–		–[d]	–
1[b,c]	<0.01[b,c]	+		–		–		+	–
1[b,c]	4[b]	–		–		+		–[d]	–
1[b,c]	3[b]	–		–		+		+	–
1[b]	24[b]	+		–		+		+	–
1[b,c]	287[b]	–		+		–		–	–
1[b,c]	110[b]	–		+		–		+	–
1[b,c]	32[b]	–		+		+		+	–
1[b,c]	70[b]	–		–		–		–	+
1[b,c]	45[b]	–		–		–		+	+
1[b,c]	10[b]	+		–		–		–[d]	+
1[b,c]	4[b]	–		–		+		–[d]	+
1[b,c]	3[b]	–		–		+		+	+
2[c,e]	<0.01[b,c]	+		–		–		+	+
–		2[b,c]	40					–	–
			160[b]						
–		1[b,c]	100[b]					+	–
+		2[b,c]	8					–[d]	–
			32[b]						
+		2[b,c]	<0.01					+	–
			~2000[b,c]						
–		ND	>>1000[c]					–	+
–		ND	>>1000[c]					+	+
+		1[b,c]	290[b]					–[d]	+
+		1[b,c]	<0.01[b]					+	+
–				1[b]	700[b]		–	–	–
–				1[b]	700[b]		–	+	–
–				ND	~1000[c]		+	–[d]	–
–				ND	~1000[c]		+	+	–
+				1[b,c]	70[b]		–	–[d]	–
+				2[b,c]	70[b]		–	+	–
+				1[b,c]	250[b]		+	–[d]	–
–				ND	>>1000[c]		–	–[d]	+
–				ND	>>1000[c]		–	+	+
+				1[b,c]	280[b]		–	–[d]	+
+				1[b,c]	300[b]		–	+	+
–				–		>1[c]	300	–	–
							~1000[c]		
–				–		ND	>>1000[c]	+	–
–				+		ND	>>1000[c]	–	–
–				+		ND	>>1000[c]	+	–
+				–		2[b,c]	20	–[d]	–
							60[b]		
+				–		1[b]	44[b]	+[f]	–
+				–		ND	500[b]	+[g]	–
+				+		>1[c]	100	–[d]	–
							400[c]		
+				+		1[c]	90[b,c]	+[f]	–
+				+		ND	>1000[c]	+[g]	–
–				–		ND	>>1000[c]	–	+
–				–		ND	>>1000[c]	+	+
+				–		1[b,c]	130[b]	–[d]	+
+				–		ND	>1000[c]	+	+
+				+		ND	>>1000[c]	+	+

[a] The conditions were as described under Experimental Procedures. (+) and (–) indicate the absence or the presence at a saturating concentration of ligands other than that studied. When present, the concentrations of the saturating ligands were as follows: $tRNA^{Val}$, 1.5–2-fold the concentration of the synthetase, 10 mM valine, 5 mM ATP, 50 mM AMP, 10 mM PP_i, and 10 mM $MgCl_2$ unless otherwise indicated, when no other ligand except that studied was present, or 4 mM $MgCl_2$ in excess when ATP and PP_i were saturating. [b] As determined by equilibrium dialysis at 4 °C. [c] As determined by equilibrium gel filtration at 25 °C, in the presence of either 0.5–2 mM valine, 0.5–2 mM ATP, 1–4 mM AMP, or 0.5–2 mM PP_i. [d] In the presence of 15 mM Na_2EDTA. [e] The excess of labeled valine eluted in the peak (and its deficiency in the trough) of the elution profile corresponded to the sum of the charged $tRNA^{Val}$ plus the enzyme·adenylate complex. [f] In the presence of stoichiometric concentration of PP_i and $MgCl_2$. [g] In the presence of 4 mM $MgCl_2$ in excess over the PP_i concentration. ND, not determined.

gether, a very high affinity was observed (K_D < 10 nM), indicating that adenylate was synthesized. The presence of PP_i also strongly decreased this K_D (4 and 3 μM in the absence and in the presence of magnesium, whereas that of AMP increased it (280 and 110 μM in the absence and in the presence of magnesium) (Figure 3B). When AMP, PP_i, and magnesium were present together, the K_D (32 μM) was intermediate between that determined in the presence of PP_i (3 μM) or AMP (110 μM) (Figure 3B). Under PP_i–ATP exchange conditions (i.e., in the presence of ATP, PP_i, and magnesium), the K_D was 24 μM (Figure 3B). Finally, the existence of one binding site for valine was confirmed by

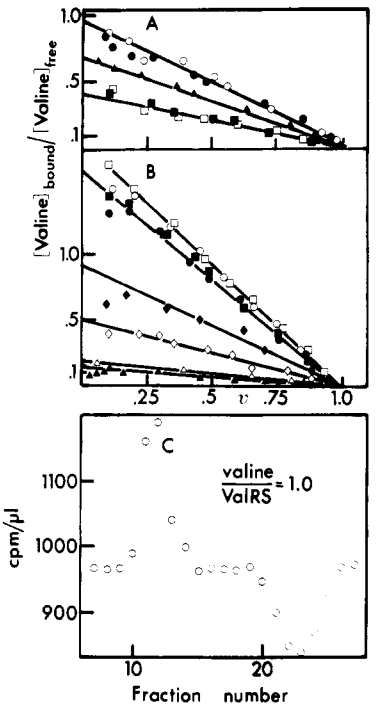

FIGURE 3: Binding of valine to valyl-tRNA synthetase studied by equilibrium dialysis at 4 °C (A and B) and equilibrium gel filtration at 25 °C (C). (A) In the absence (□) or in the presence (○) of $MgCl_2$; in the presence of $tRNA^{Val}$ with (●) or without (■) $MgCl_2$; in the presence of ATP plus Na_2EDTA (▲). When present, the reactants were at following concentrations: 10 or 4 mM $MgCl_2$ in excess over ATP; 15 mM Na_2EDTA; 5 mM ATP; 55 μM $tRNA^{Val}$; various concentrations of valine [10–510 μM (■, □), 10 μM to 2 mM (●, ○), 1–85 μM (▲)] and of valyl-tRNA synthetase [28 (■, □), 43 (●, ○), 6 μM (▲)]. (B) In the presence of PP_i (●), PP_i plus $MgCl_2$ (○), PP_i plus $tRNA^{Val}$ (■), PP_i plus $tRNA^{Val}$ plus $MgCl_2$ (□), AMP (▲), AMP plus $MgCl_2$ (△), AMP plus PP_i plus $MgCl_2$ (♦), and ATP plus PP_i plus $MgCl_2$ (◇). When present, the reactants were at following concentrations: 10 mM PP_i; 50 mM AMP; 5 mM ATP; 10 μM $tRNA^{Val}$; 4 mM $MgCl_2$ in excess over the sum of ATP plus PP_i; 1–60 μM (○, ●, □, ■), or 7 μM–1.5 mM (▲), 7–460 (△), or 6–520 (♦), or 4–200 μM (◇) valine; 6 (○, ●, □, ■), 40 (▲), 23 (△), 28 (♦), or 12 μM (◇) valyl-tRNA synthetase. (C) In the presence of PP_i and $MgCl_2$. The equilibrium buffer contained 10 mM PP_i, 14 mM $MgCl_2$, and 200 μM [^{14}C]valine (4.8 cpm/pmol). The 100-μL sample contained 5000 pmol of valyl-tRNA synthetase; fractions of ~50 μL were collected. The stoichiometry of the enzyme-bound valine, determined from the peak and trough of the elution profile, is indicated.

equilibrium gel filtration of the synthetase in the presence of PP_i (Figure 3C).

The presence of $tRNA^{Val}$ did not affect the ATP- and PP_i-promoted decreases of the K_D of valine for the synthetase. In the presence of ATP the K_D was 10 μM in the absence of magnesium or lower than 10 nM in the presence of magnesium (under adenylate synthesis conditions). In the presence of PP_i, the K_D was 3 or 4 μM in the absence or in the presence of magnesium (Figure 3B). Finally, in the presence of all substrates (ATP, $MgCl_2$, valine, and $tRNA^{Val}$), two valine moieties were found bound on the synthetase: a first one as Val~AMP and a second one as Val-$tRNA^{Val}$.

(*4*) *ATP.* (See Table II and Figure 4.) In the absence of magnesium two binding sites for ATP were detected on the synthetase either by equilibrium dialysis (Figure 4A) or by equilibrium gel filtration (Figure 4A′) (K_D = 40 and 160 μM, respectively, at 4 °C and pH 7.5). However, in the presence of 10 mM magnesium, only one binding site could be detected (K_D = 100 μM at 4 °C and 60 μM at 25 °C, pH 7.5; Figure 4B). In the presence of valine, using ATP labeled on its AMP moiety, we detected two binding sites for the nucleotide either in the absence of magnesium with Na_2EDTA so that the synthesis of adenylate is hindered (K_D = 8 and 32 μM; Figure 4C) or in its presence (one as adenylate with a K_D < 10 nM and another one as ATP with a $K_D \simeq 2$ mM; Figure 4C′).

The presence of $tRNA^{Val}$ resulted in a strong increase of the K_D of ATP for the synthetase and in the suppression of one of the two binding sites. When valine was absent, the K_D was higher than 1 mM whether magnesium was present or nor (Figure 4D), whereas in the presence of valine the K_D was 290 μM in the absence of magnesium and lower than 10 nM in its presence (i.e., under adenylate synthesis conditions).

Valyl-tRNA synthetase was also found to be able to bind other nucleoside triphosphates such as GTP and CTP. Valine promoted these bindings. Figure 4E shows that similar to that observed for ATP, the synthetase bound two GTP or two CTP in the presence of valine and in the absence of magnesium but only one of these nucleotides when magnesium was present.

(*5*) *AMP.* (See Table II and Figure 5A–C.) In the absence of the other ligands one AMP was bound per enzyme (K_D = 700 μM either in the absence or in the presence of magnesium; Figure 5A). The presence of PP_i increased this K_D to ~1 mM; valine on the contrary decreased it (K_D = 70 μM either in the absence or in the presence of magnesium). However, whereas only one binding site was detected in the presence of valine and in the absence of magnesium, two equivalent sites were detected in the presence of both valine and magnesium (Figure 5B,C). In the presence of valine and PP_i, but in the absence of magnesium, one binding site was detected (K_D = 250 μM).

The presence of $tRNA^{Val}$ increased significantly the K_D of AMP for the synthetase whether the other ligands were present or not (in the absence of the small ligands, the K_D was much higher than 1 mM, whereas in the presence of valine it was ~300 μM, whether magnesium was present or not). Finally, only one AMP binding site could be detected in the presence of $tRNA^{Val}$ under the conditions where two sites were titrated in its absence (valine being present and magnesium absent; Figure 5B).

(*6*) *PP_i.* (See Table II and Figure 5D,D′.) In the absence of magnesium two binding sites for PP_i were detected on the synthetase (at 4 °C and pH 7.4 the K_D values were 300 μM and higher than 1 mM); in the presence of the cation, no stoichiometry could be determined because of the low affinity of PP_i ($K_D \gg 1$ mM) for the enzyme. In the presence of AMP and either in the absence or in the presence or magnesium, the K_D was significantly higher than 1 mM. Valine decreased the K_D of PP_i for the synthetase; two binding sites were detected in the absence of magnesium (K_D = 20 and 60 μM) but only one in its presence (K_D = 44 μM when PP_i and magnesium were present in stoichiometric concentrations and 500 μM in the presence of a large excess of magnesium). When valine and AMP were present together, two binding sites were detected in the absence of magnesium (K_D = 100 and 400 μM) but only one in its presence (K_D = 90 μM when PP_i and magnesium were present in stoichiometric concentrations and higher than 1 mM in the presence of a large excess of magnesium). Finally, $tRNA^{Val}$ increased strongly the K_D of PP_i for the synthetase: under the conditions tested the K_D was higher than 1 mM except in the presence of valine and in the absence of magnesium, where it was equal to 130 μM for a unique site.

Properties of Enzyme-Bound Adenylate. The enzyme·Val~AMP complex was isolated on Sephadex G-75 with a yield of ~100%. When separated from the excess of ligands, it exhibited an unusually high stability compared to that of other adenylate complexes [i.e., Baltzinger & Remy (1977)]. Actually, it could be stored for several days at 0 °C (in 50

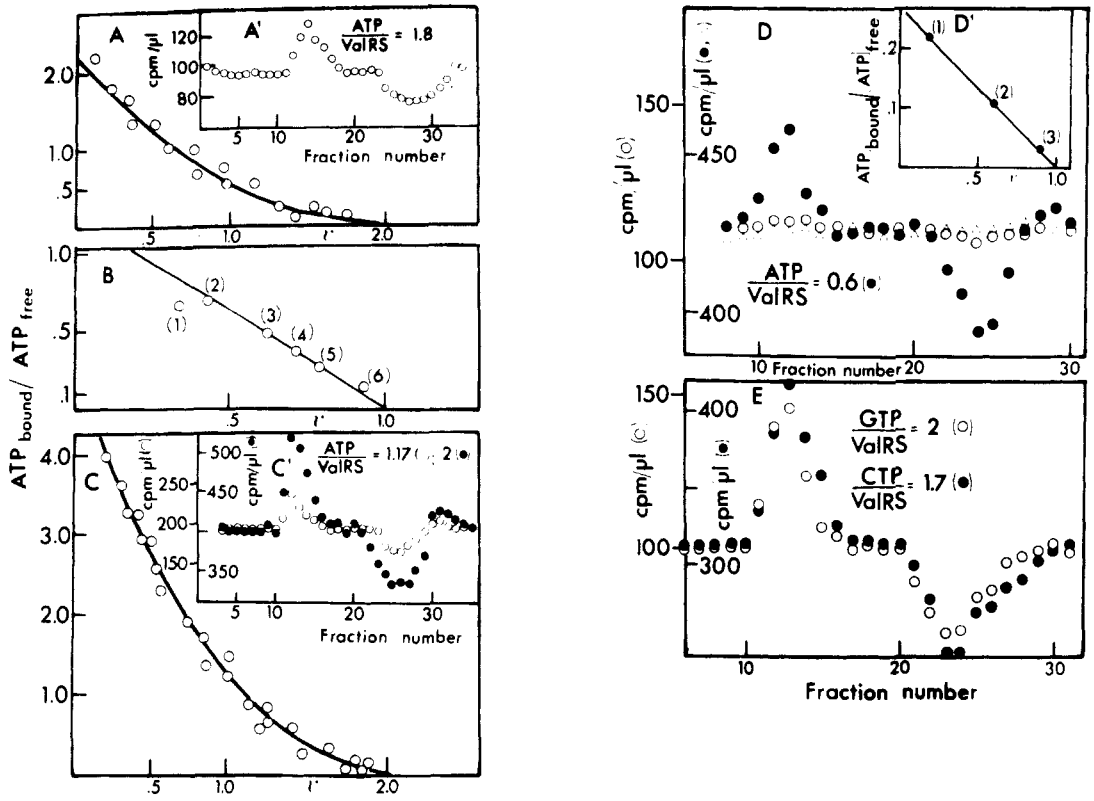

FIGURE 4: Binding of ATP (A–D) and GTP or CTP (E) to valyl-tRNA synthetase studied by equilibrium dialysis and equilibrium gel filtration. (A and A′) In the absence of magnesium as studied by equilibrium dialysis at 4 °C (A) and equilibrium gel filtration at 25 °C (A′). (A) The concentration of ATP varied from 6 μM to 1 mM, and that of valyl-tRNA synthetase was 46 μM. The solid line (—) represents the calculated binding curve (by replacing K_1 and K_2 in eq 2 by 40 and 160 μM, respectively) and (O) the experimental points. (A′) The equilibrium buffer contained 440 μM [^{14}C]ATP (0.215 cpm/pmol), and 12 400 pmol of valyl-tRNA synthetase was in the 100-μL sample; ~45-μL fractions were collected. The stoichiometry of the enzyme-bound ATP, determined from the peak and trough of the elution profile, is indicated. (B) In the presence of magnesium as studied by equilibrium gel filtration at 4 °C. The equilibrium buffer contained 10 mM $MgCl_2$ and the following concentrations of [^{14}C]ATP (2.1 cpm/pmol): (1) 50, (2) 75, (3) 160, (4) 250, (5) 360, and (6) 1400 μM. 12 000 pmol of valyl-tRNA synthetase was in the 100-μL sample. (C and C′) In the presence of valine without [C and C′ (●)] or with [C′ (O)] magnesium as studied by equilibrium dialysis at 4 °C (C) and equilibrium gel filtration at 25 °C (C′). In (C) ATP varied from 3 to 500 μM; 10 mM L-valine, 15 mM Na_2EDTA, and 20 μM enzyme were present. The solid line (—) represents the calculated binding curve (by replacing K_1 and K_2 in eq 2 by 8 and 32 μM, respectively) and (O) the experimental points. (C′) (O) the equilibrium buffer contained 480 μM [^{14}C]ATP (0.4 cpm/pmol), 10 mM L-valine, and 4 mM $MgCl_2$; 10 000 pmol of valyl-tRNA synthetase was present in the 100-μL sample; ~43-μL fractions were collected. (●) The equilibrium buffer contained 480 μM [^{14}C]ATP (0.84 cpm/pmol), 10 mM L-valine, and 15 mM Na_2EDTA; 11 100 pmol of valyl-tRNA synthetase was present in the 100-μL sample; ~53-μL fractions were collected. The stoichiometry of the enzyme-bound ATP, determined from the peak and trough of the elution profile, is indicated. (D and D′) In the presence of $tRNA^{Val}$ and without (O) or with (Δ) magnesium (D) and in the presence of $tRNA^{Val}$, valine, and Na_2EDTA (●) (D and D′) as studied by equilibrium gel filtration at 25 °C. (O) The equilibrium buffer contained 200 μM [^{14}C]ATP (0.55 cpm/pmol); 6800 pmol of valyl-tRNA synthetase and 8500 pmol of $tRNA^{Val}$ were in the 100-μL sample. (Δ) The equilibrium buffer contained 450 μM [^{14}C]ATP (0.95 cpm/pmol) and 4 mM $MgCl_2$; 7900 pmol of valyl-tRNA synthetase and 8500 pmol of $tRNA^{Val}$ were in the 100-μL sample. (●) The column was equilibrated with 75 [D′(1)], 450 [D and D′(2)], or 2300 μM [D′(3)] [^{14}C]ATP (0.95 cpm/pmol), 10 mM L-valine, and 15 mM Na_2EDTA; 7900 pmol of valyl-tRNA synthetase and 8500 pmol of $tRNA^{Val}$ were in the 100-μL sample; ~42-μL fractions were collected. The stoichiometry of the enzyme-bound ATP, determined from the peak and trough of the elution profile of experiment (●), is indicated. (E) Binding of GTP (O) and CTP (●) as studied by equilibrium gel filtration at 25 °C. The equilibrium buffer contained 200 μM [α-^{32}P]GTP (0.5 cpm/pmol) or 200 μM [^{14}C]CTP (1.55 cpm/pmol), 10 mM L-valine, and 15 mM Na_2EDTA; 5400 pmol of valyl-tRNA synthetase was in the 100-μL sample, and ~47-μL fractions were collected. The stoichiometries of the enzyme-bound GTP and CTP, determined from the peak and trough of the elution profile, are indicated.

mM NaHepes, pH 7.0, or 50 mM Tris-HCl, pH 7.4) without modification of its capacity to transfer the activated valine to $tRNA^{Val}$ (yield ~90%). At 37 °C the half-life of the complex was higher than 9 h (in 100 mM Tris-HCl, pH 7.4, and either 2 or 20 mM $MgCl_2$). However, incubation in the presence of a low ionic strength decreased its stability (half-life ~3 h at 37 °C in 10 mM Tris-HCl, pH 7.4, and 2 mM $MgCl_2$). Zinc acetate also destabilized the complex; at 37 °C in 100 mM Tris-HCl, pH 7.4, half-lifes of 120, 50, and 10 min were determined in the presence of 10 μM, 100 μM, and 1 mM zinc, respectively. A K_m value of 3 mM for the zinc-promoted effect could be determined (not shown). Under similar incubation conditions, magnesium had no effect and did not influence the zinc-promoted instability of the complex.

(1) Modification of the Association Parameters of the Enzyme·Adenylate Complex during Storage. The storage of the complex resulted in a decrease of the strength of interaction of Val~AMP with the synthetase. After a 1-week storage the complex was found in equilibrium with free enzyme and adenylate, and it became more sensitive to the destabilizing agents (i.e., temperature, dilution, zinc, magnesium, low ionic strength), but its transfer capacity of valine to $tRNA^{Val}$ remained almost unaffected.

The demonstration for the establishment of an equilibrium between enzyme-bound and free adenylate during storage of the complex was done by dilution experiments of a 1-week-old complex. Such dilutions resulted in a decrease of its yield of retention on nitrocellulose disks. Whereas at 0 °C, after a rapid decrease consecutive to the dilution, the amount of adenylate retained on the disks remained constant (Figure 6A,A′), at 37 °C the kinetics of disappearance of the retained adenylate were biphasic (Figure 6B) with a first fast phase

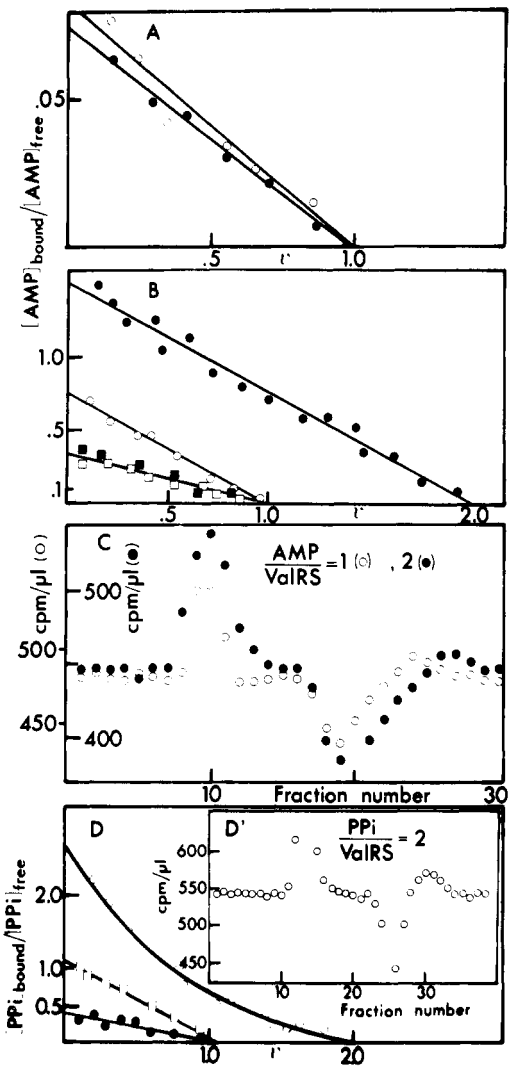

FIGURE 5: Binding of AMP (A–C) and PP_i (D) to valyl-tRNA synthetase studied by equilibrium dialysis and equilibrium gel filtration. (A) In the presence (○) or in the absence (●) of magnesium as studied by equilibrium dialysis at 4 °C. AMP varied from 0.125 to 4 mM, enzyme was 60 μM, and, when present, magnesium was 10 mM. (B) In the presence of valine (○, ●) and in that of valine plus $tRNA^{Val}$ (□, ■) either without (○, □) or with (●, ■) magnesium as studied by equilibrium dialysis at 4 °C. AMP varied from 14 μM to 2.5 mM (○, ●) or from 20 μM to 2 mM (□, ■); either 4 mM $MgCl_2$ (●, ■) or 15 mM Na_2EDTA (○, □) was present; valyl-tRNA synthetase was 55 (○, ●) or 100 μM (□, ■). (C) In the presence of valine without (○) or with (●) magnesium as studied by equilibrium gel filtration at 25 °C. The equilibrium buffer contained 500 μM [^{14}C]AMP [0.96 cpm/pmol (○) or 0.88 cpm/pmol (●)], 10 mM L-valine, and either 4 mM $MgCl_2$ (●) or 15 mM Na_2EDTA (○); 7500 pmol of valyl-tRNA synthetase was in the 100-μL sample; ~55-μL (○) or 38-μL (●) fractions were collected. (D) In the presence of valine without (○) or with (□) magnesium and in the presence of valine and $tRNA^{Val}$ (●) as studied by equilibrium dialysis at 4 °C. PP_i varied from 6 to 600 (○), from 5 to 1400 (□), or from 15 to 1700 μM (●); valine was 10 mM, and, when present, magnesium was stoichiometric with PP_i; valyl-tRNA synthetase was 24 (○) or 48 μM (□, ●), and, when present, $tRNA^{Val}$ was 30 μM. The solid line (—) represents the calculated binding curve (by replacing K_1 and K_2 in eq 2 by 20 and 60 μM, respectively) and (○) the experimental points. (D′) In the presence of valine without magnesium as studied by equilibrium gel filtration at 25 °C. The equilibrium buffer contained 400 μM [^{32}P]PP_i (1.35 cpm/pmol), 10 mM L-valine, and 15 mM Na_2EDTA; the 100-μL sample contained 10 960 pmol of valyl-tRNA synthetase; ~47-μL fractions were collected. The stoichiometry of the enzyme-bound PP_i, determined from the peak and trough of the elution profile, is indicated.

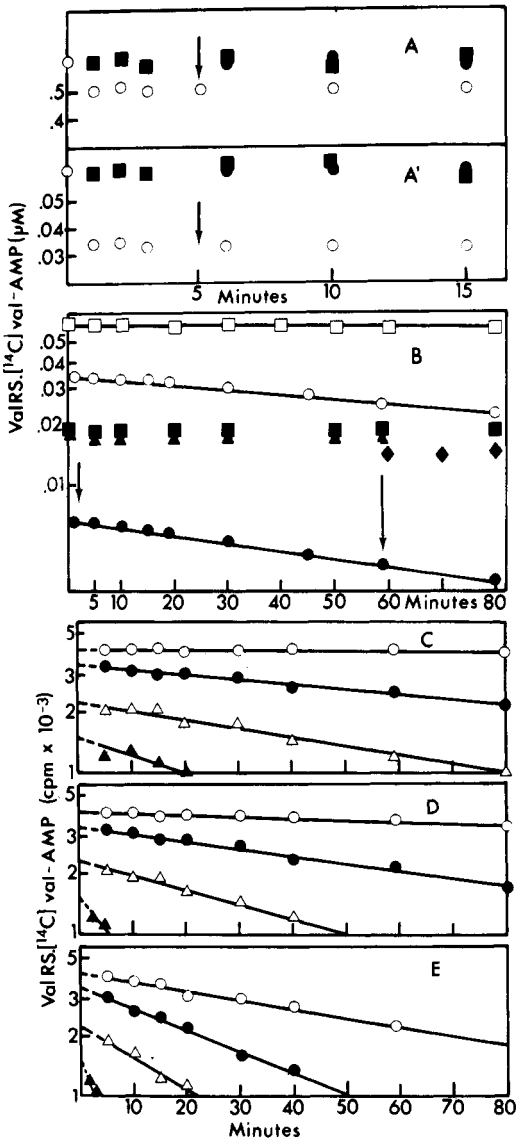

FIGURE 6: Stability of the enzyme·adenylate complex after a 1-week storage at 0 °C. A 4.6 μM stock solution of enzyme·[^{14}C]Val~AMP complex was stored 1 week at 0 °C before dilution. (A and B) Effect of high dilutions at 0 °C (A and A′) or 37 °C (B). (A and A′) Dilutions of the complex to a final concentration of 0.62 (A) or 0.062 μM (A′) in 100 mM NaHepes, pH 7.0, containing 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, and either no (○) or 10 μM (■) valyl-tRNA synthetase. The arrows indicate the addition of 10 μM enzyme in the incubation mixture under conditions (○), and (●) indicates the recovering of the complex. (B) Dilutions of the complex at 37 °C to a final concentration of 0.062 (□, ○) or 0.018 μM (●, ■, ▲, ♦) in the same buffer as in (A) and (A′) either not containing (○, ●) or containing 10 μM (□, ■) valyl-tRNA synthetase. The arrows indicate the addition of 10 μM enzyme in the incubation mixture under conditions (●), and (▲, ♦) indicate the recovery of the complex. (C–E) Effect of high dilutions at different pHs at 37 °C. The complex was diluted to a final concentration of 0.62 (○), 0.062 (●), 0.018 (△), or 0.009 μM (▲) in 100 mM NaHepes, pH 7.0 (C), 8.0 (D), or 9.0 (E), containing 5 mM β-mercaptoethanol and 2 mM $MgCl_2$. After various incubation times, aliquots of relative volumes, respectively, 1 (○), 10 (●), 33 (△), and 66 (▲) were filtered through nitrocellulose disks. The retained material was determined as described under Experimental Procedures.

followed by a much slower one whose rate was of the first order. Increasing dilutions increased the amplitude of the first phase and the rate of the second one (Figure 6B). The presence of an excess of free enzyme reduced strongly the dilution-promoted destabilization of the adenylate complex. At 0 °C the highly diluted complex could be quantitatively retained on the disks in the presence of a large excess of free enzyme, whether the enzyme was added in the dilution buffer before or after the complex (Figure 6A,A′). At 37 °C, however, this protection occurred the most efficiently when the

Table III: Determination of the Dissociation Constant (K_D) of the Valyl-tRNA Synthetase·Val~AMP Complex[a]

	initial state		final state		
expt	complex [A] (μM)	free enzyme added (μM)	complex [B] (μM)	free enzyme (μM)	K_D[b]
1	0.62	0	0.51	0.11	0.023
2	0.62	10	0.62	10	
3	0.062	0	0.034	0.028	0.023
4	0.062	10	0.062	10	
5	0.018	0	0.0066	0.012	0.022
6	0.018	10	0.018	10	
7	0.009	0	0.0023	0.007	0.021
8	0.009	10	0.009	10	

[a] The [^{14}C]valine-labeled complex (stock solution, 4.6 μM in a 1 to 1 stoichiometric ratio) was stored 1 week at 0 °C before use. It was then diluted (final concentration = [A]) in 100 mM NaHepes, pH 7.0, containing 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, and either 10 μM valyl-tRNA synthetase (experiments 2, 4, 6, and 8) or no free enzyme (experiments 1, 3, 5, and 7). At various incubation times from 1 to 15 min at 0 °C, aliquots were filtered through nitrocellulose disks, and the remaining enzyme·Val~AMP complex ([B]) was determined. [b] Since the yield of retention of the adenylate complex on the nitrocellulose disks was 100%, the concentrations of free adenylate (dissociated from enzyme) and free enzyme (in the absence of addition of enzyme in the incubation mixture) correspond to [C] = [A] − [B]. Thus $K_D = [C]^2/[B]$.

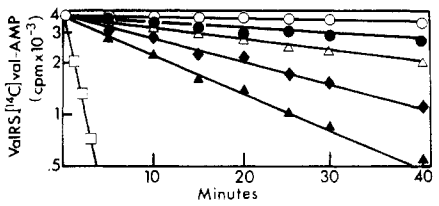

FIGURE 7: Effect of the ligands on the stability of the enzyme·adenylate complex at 37 °C. The freshly prepared complex (4.6 μM) was diluted as described in Figure 6 to a final concentration of 0.46 μM; the dilution buffers contained either no substrates (○) or 1 mM L-valine (●), 1 mM ATP (△), 1 mM L-valine plus 1 mM ATP (◆), 1 μM $tRNA_{ox}^{Val}$ (▲), or 1 mM L-valine plus 1 mM ATP plus 1 μM $tRNA_{ox}^{Val}$ (□). For prevention of the pyrophosphorolysis of the adenylate by contaminating PP_i, the incubation mixture was preincubated before addition of the enzyme·adenylate complex in the presence of pyrophosphatase (2 units/mL) for 15 min at 37 °C.

enzyme was added before the complex in the dilution buffer (Figure 6B). On the other hand, increasing incubation times of the highly diluted complex at 37 °C decreased the efficiency of the free enzyme to reverse the dilution-promoted destabilization of the complex (Figure 6B). Since free adenylate is not retained on nitrocellulose disks, these results suggest the existence of an equilibrium between enzyme-bound and free adenylate, the free adenylate being thermolabile. A dissociation constant value (K_D) of 22 nM could be determined at 0 °C for the enzyme·Val~AMP complex (Table III). Since no dissociation occurred on the freshly prepared complex, its K_D is significantly lower than 22 nM. This modification affects specifically the adenylate site of the synthetase because its specific activity for tRNA charging remained unchanged during storage and because this change did not affect the transfer of the activated valine to $tRNA^{Val}$. This equilibrium was not significantly affected by pH changes contrary to the stability of the free adenylate which decreased with increasing pHs. This emerges from the biphasic dilution-promoted dissociation kinetics of the complex at pH 7.0, 8.0, and 9.0 shown in Figure 6C–E: the amplitudes of the first fast phases were independent of pH, whereas the rates of the second slow phases increased with increasing pHs.

Table IV: Effect of pH on the First-Order Rate Constant of Hydrolysis of the Valyl-tRNA Synthetase·Val~AMP Complex (k_1) in the Absence and in the Presence of $tRNA_{ox}^{Val}$ and on the Rate Constant of $tRNA^{Val}$ Charging (k_2)

	k_1[a] (×10^3 min^{-1})		
pH	$-tRNA_{ox}^{Val}$	$+tRNA_{ox}^{Val}$	k_2[b] (min^{-1})
6.2	2.25	2.65	96
7.0	2.0	53.0	450
8.0	6.6	231.0	690
9.0	17.0	462.0	252

[a] The freshly prepared enzyme·[^{14}C]Val~AMP complex was diluted (final concentration, 0.5 μM) in 100 mM NaHepes buffer, at the indicated pH, containing 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, and, when present, 1 μM $tRNA_{ox}^{Val}$. After various incubation times at 37 °C the remaining complex was determined, and the rate constants of hydrolysis was then calculated as described under Experimental Procedures. [b] The rate constants of $tRNA^{Val}$ charging were determined at 37 °C in 100 mM NaHepes buffer at the indicated pH and in the presence of saturating concentrations of substrates (0.2 mM [^{14}C]valine, 10 mM ATP, and 20 μM $tRNA^{Val}$) as described under Experimental Procedures.

Table V: Comparison of the Ability of Various $tRNAs_{ox}$ To Destabilize the Valyl-tRNA Synthetase·Val~AMP Complex and the Ability of the Accepting tRNAs To Be Aminoacylated by Valyl-tRNA Synthetase

$tRNA_{ox}$ specific for	k_1[a] (×10^3 min^{-1})	k_2 (relative values)[b]
	31	
Val	690	100
Met	70	0.1
Phe	40	0.02
Gly	41	0.02
Leu	38	0.003
Arg	35	0.004
Asp	35	0.004

[a] The freshly prepared enzyme·[^{14}C]Val ~AMP complex was diluted to a final concentration of 0.5 μM at 37 °C in 100 mM NaHepes, pH 7.0, containing 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM ATP, 1 mM L-valine, and 10 μM specific $tRNA_{ox}$. The first-order rate constants of hydrolysis (k_1) were calculated as described under Experimental Procedures. [b] Relative rates of tRNA charging by valyl-tRNA synthetase (k_2) under standard aminoacylation conditions.

(2) Effect of Substrates and of pH on the Stability of the Freshly Isolated Enzyme·Val~AMP Complex. At 37 °C all ligands destabilized the complex (Figure 7). The presence of valine and ATP decreased its half-life about 2 and 4 times, respectively, whereas their copresence decreased it ~8 times (under the experimental conditions used the half-life of the complex was 200 min in the absence of ligands, and 90, 45, and 22 min in the presence of valine, ATP, and valine plus ATP, respectively). $tRNA_{ox}^{Val}$ decreased strongly the stability of the complex (half-life of 13 min). When all ligands (ATP, valine, and $tRNA_{ox}^{Val}$) were present, the complex was ~200 times less stable than in their absence (half-lifes of 200 and 1 min, respectively; Figure 7).

The pH affects the $tRNA_{ox}^{Val}$-promoted instability of the complex. Table IV shows that increasing pH from 6.2 to 8.0 increases the rates of the $tRNA_{ox}^{Val}$-promoted hydrolysis of the adenylate and of $tRNA^{Val}$ charging. This parallel pH effects on both reactions, however, did not occur at higher pH values, since from pH 8.0 to 9.0 the $tRNA_{ox}^{Val}$-promoted hydrolysis of the complex continued to increase, whereas the charging of unmodified $tRNA^{Val}$ decreased. This last effect results from a strong deacylation of Val-$tRNA^{Val}$ and/or from the displacement of the rate-determining step of the $tRNA^{Val}$ charging process.

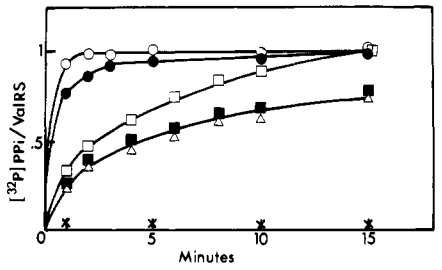

FIGURE 8: Stimulation of the cleavage of [γ-^{32}P]ATP by valyl-tRNA synthetase in the presence of various amino acids. The reaction mixtures contained 100 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 units of pyrophosphatase/mL, 22 μM [γ-^{32}P]ATP (142 cpm/pmol), 2.95 μM valyl-tRNA synthetase, and either 5 mM L-valine (○), L-threonine (●), L-cysteine (□), L-isoleucine (■), or L-alanine (△) or no amino acid (×). The [^{32}P]PP_i released after various incubation times at 0 °C was determined as described under Experimental Procedures.

Finally, Table V shows that noncognate $tRNAs_{ox}$ also destabilized the adenylate complex. Interestingly, there is a good correlation between the ability of a tRNA to be charged by the synthetase and its ability to destabilize after periodate oxidation the adenylate complex.

(3) Activation of Other Amino Acids by Valyl-tRNA Synthetase and Stability of the Enzyme-Bound Adenylates Involving Misactivated Amino Acids. Apart from valine four other amino acids (alanine, cysteine, isoleucine, and threonine) were found to be able to stimulate the [^{32}P]PP_i–ATP exchange catalyzed by valyl-tRNA synthetase (kinetic parameters were only measured for threonine: k_{cat} = 30 s^{-1} and K_m = 7 mM compared to k_{cat} = 60 s^{-1} and K_m = 50 μM for valine). These wrong amino acids, especially threonine, promoted the amino acid dependent cleavage of [γ-^{32}P]ATP (Figure 8). The valyl-tRNA synthetase·Thr~AMP complex could be isolated on Sephadex G-75 with a yield of 70%, indicating that Thr~AMP formed a less stable complex with the synthetase than Val~AMP did. The enzyme·Thr~AMP complex was more destabilized by the factors studied previously on the enzyme·Val~AMP complex; for example, at 37 °C in the presence of 50 mM NaHepes, pH 7.0, and 2 mM $MgCl_2$, the wrong complex was ~15 times less stable than the correct one (Table VI). When present individually, ATP and valine destabilized the two complexes by a same factor (2–4), whereas in their copresence the wrong complex was ~10 times less stable than the correct one. Either in the absence or in the presence of the small ligands, $tRNA_{ox}^{Val}$ destabilized the wrong complex ~3 times more than the correct one (Table VI). Finally in the presence of 100 μM zinc the half-life of the wrong complex was ~10 times lower than that of the correct one.

Structural State of Valyl-tRNA Synthetase under Noncatalytic and Catalytic Conditions. In the absence of the substrates, valyl-tRNA synthetase and purified $tRNA^{Val}$ sedimented in a 5–20% sucrose gradient similarly to globular proteins of molecular weight, respectively, 102 000 and 55 000. When unfractionated tRNA was submitted to centrifugation, the $tRNA^{Val}$ sedimented with an apparent molecular weight of 87 000 indicating the existence of dimers between $tRNA^{Val}$ and another specific tRNA (see Experimental Procedures). A similar apparent molecular weight has been determined for the dimers of $tRNA^{Glu}$ (Kern & Lapointe, 1979a). When the gradient was equilibrated with ATP and valine (under adenylate synthesis conditions), valyl-tRNA synthetase sedimented with an apparent molecular weight of 102 000. With $tRNA^{Val}$ along the gradient the synthetase·$tRNA^{Val}$ complex had an apparent molecular weight of 176 000. The same molecular weight was found under tRNA charging conditions, when the gradient was equilibrated with the three substrates $tRNA^{Val}$, ATP, and valine.

Table VI: Comparison of the Half-Lifes of the Valyl-tRNA Synthetase Bound Val~AMP and Thr~AMP Complexes under Various Experimental Conditions at 37 °C[a]

substrates			half-lifes	
$tRNA_{ox}^{Val}$	ATP	Val	ValRS·Val~AMP (min)	ValRS·Thr~AMP (min)
–	–	–	~200	13
–	–	+	90	4
–	+	–	45	3
–	+	+	22	0.17
+	–	–	13	0.33
+	+	+	1	<0.03

[a] The freshly prepared complexes, ^{14}C-labeled on the amino acid, were diluted to a final concentration of 0.5 μM in 50 mM NaHepes, pH 7.0, containing 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, and, when present, 1 mM ATP, 1 mM L-valine, and 1 μM $tRNA_{ox}^{Val}$.

Discussion

The active-site titration and the direct binding experiments reported here show that yeast valyl-tRNA synthetase possesses only one adenylation site and one binding site for $tRNA^{Val}$. There is also one valine binding site on the free enzyme, but there are two sites for ATP (or for GTP or CTP). Since the free enzyme also binds two PP_i but only one AMP, it appears that in one of the two binding sites (most probably the specific one) both AMP and PP_i moieties of ATP interact with the synthetase, whereas in the second site the PP_i moiety alone is likely involved.

Relation between the Binding Sites of the Small Ligands. (1) Valine and ATP or PP_i. Our results show a reciprocal synergistic effect in the binding of valine and ATP with valyl-tRNA synthetase, even under conditions where no adenylate is formed, indicating a coupling between both binding sites (Table II). Such a coupling has also been reported for *Escherichia coli* isoleucyl- and phenylalanyl-tRNA synthetases (Holler et al., 1973a,b) and for *E. coli* methionyl-tRNA synthetase (Fayat & Waller, 1974). This relation exists for both ATP interacting sites. Since we also found a strong reciprocal synergism between the binding of valine and PP_i but a hindrance induced by AMP for that of valine, it appears that the coupling between ATP and valine is in fact mediated by the PP_i moiety of ATP.

(2) Valine and AMP. The relation between the binding sites of AMP and valine is of a higher degree of complexity, since there is no reciprocal synergistic effect in their binding: a saturating concentration of AMP hinders the binding of valine at a limiting concentration, whereas at a saturating concentration, valine increases the affinity of AMP for the synthetase (Table II). These properties can be explained by assuming that both ligands combine with the free synthetase and generate different enzyme forms; valine binds more readily to the free enzyme than to the enzyme·AMP complex (which prevails when AMP is saturating and valine limiting), whereas AMP binds more readily to the enzyme·Val complex than to the free enzyme (which prevails when valine is saturating and AMP limiting). However, since both the free enzyme and the enzyme·Val complex possess only one binding site for AMP, the same region of the synthetase is involved in the interaction with this ligand; probably, the association of AMP with the free synthetase generates an abortive complex unable to combine productively valine, whereas valine generates an enzyme form (E*) bestowed with the specific binding site for AMP (eq 3)

$$E + AMP \rightarrow E{\cdot}AMP \xrightarrow{+Val} E{\cdot}AMP{\cdot}Val$$

$$E + Val \rightarrow E^{*}{\cdot}Val \xrightarrow{+AMP} E^{*}{\cdot}Val{\cdot}AMP \qquad (3)$$

The considerably higher affinity of Val~AMP for the synthetase (at least 1000-fold) as compared to valine and AMP (or ATP) either individually or together (Table II) indicates the existence of a strong synergism between the AMP and the valine parts in the binding of Val~AMP. This synergism cannot be simulated by binding free AMP and valine to the synthetase. This is probably a consequence of steric hindrances when both ligands are individually bound on the enzyme. On the other hand, it could be that free valine and AMP are unable to generate the enzyme form involved in adenylate synthesis.

(*3*) *AMP and PP_i.* ATP has a much higher affinity for the synthetase than AMP and PP_i either individually or together. Here also there is a strong synergism between the AMP and PP_i moieties in the interaction of ATP with the enzyme which cannot be simulated, due to steric hindrances, by binding the free ligands individually. Steric hindrances explain also the decrease of the synergism between valine and PP_i when AMP is present and between valine and AMP when PP_i is present (Table II).

(*4*) *Effect of Magnesium.* The presence of the bivalent cation results in the suppression of one ATP (and PP_i) interacting site of the synthetase and in a strong decrease of the affinity of the second one for these ligands. This may partly be ascribed to a direct contribution of the phosphate groups of ATP and PP_i to the binding, which is abolished or strongly reduced when ATP and PP_i acquire a constrained conformation upon chelation with magnesium (Glassman et al., 1971). A similar magnesium effect on the binding of ATP has been reported for the *E. coli* methionyl-tRNA synthetase (Fayat & Waller, 1974). The suppression of the second ATP site by magnesium indicates that this ligand interacts differently on its two binding sites: in one site (probably the specific one) both AMP and PP_i moieties of ATP are involved in the interaction, whereas in the other site, the PP_i moiety of ATP is rather involved. This mode of binding of ATP is supported by the fact that in the absence of magnesium, GTP and CTP also combine with the synthetase in a 2 to 1 stoichiometric ratio, whereas AMP combines in a 1 to 1 ratio. Since one PP_i binding site is also suppressed in the enzyme·Val complex, whereas the coupling between the sites of valine and of PP_i is maintained, another specific effect of magnesium on the synthetase, which decreases its affinity for $PP_i{\cdot}MgCl_2$, may occur.

Our experiments bring evidence that a second $ATP{\cdot}MgCl_2$ molecule interacts with the enzyme·adenylate complex, but with a considerably lower affinity than the ATP involved in the adenylate. They also show that the enzyme·Val complex binds two AMP but only one PP_i in the presence of magnesium and one AMP and two PP_i in the absence of the cation. It appears thus that valine promotes and magnesium modulates the interaction of AMP and PP_i (either free or as parts of ATP) with the second ATP binding site: either PP_i or AMP (free or as parts of the nucleoside triphosphates) reacts preferentially at this site, magnesium being present or not.

It has been shown that magnesium ions are not essential for the formation of aminoacyladenylate in the *E. coli* isoleucine and methionine systems but that they accelerate considerably its rate of formation (Cole & Schimmel, 1970; Blanquet et al., 1972); this likely applies also to other amino acid activating systems such as the valine one. The fact that $ATP{\cdot}MgCl_2$ is the catalytically active species is in apparent contradiction with the higher affinity of ATP as compared to that of $ATP{\cdot}MgCl_2$ for the synthetase. This can be related to different modes of interaction of ATP with its specific binding site, whether magnesium and valine are present or not. Since in the presence of magnesium, AMP has a higher affinity for the synthetase than does PP_i, it is likely that $ATP{\cdot}MgCl_2$ interacts with the enzyme first with its AMP part. However, this interaction was shown to be abortive (eq 3). It must thus be assumed that the interaction of the $PP_2{\cdot}MgCl_2$ part with the enzyme promotes and strengthens the correct interaction of the AMP part. In the absence of magnesium, AMP and PP_i exhibit similar affinities for the synthetase, suggesting that ATP interacts first with either its AMP or its PP_i part. In the presence of valine, whether magnesium is present or not, PP_i binds more readily with the synthetase than does AMP. Finally, when valine and magnesium are present together (i.e., when the second PP_i interacting site is suppressed), GTP and CTP bind also to the enzyme. This suggests that ATP or $ATP{\cdot}MgCl_2$ (and more generally the nucleoside triphosphates) interact with the enzyme first and preferentially with their PP_i or $PP_i{\cdot}MgCl_2$ moiety; the binding of the AMP moiety of ATP to the synthetase is secondly promoted after binding of valine. This emerges from the fact that in the absence or in the presence of magnesium, valine increases the affinity of AMP for the synthetase (Table II).

(*5*) *Effect of $tRNA^{Val}$.* Contrary to that observed in other tRNA acylation systems, the cognate tRNA does not affect the affinity of the amino acid for the synthetase in the valine system (Table II). For instance, in the yeast arginine (Fersht et al., 1978) and the *E. coli* glutamic acid systems (Kern & Lapointe, 1979a), the cognate tRNA promotes the binding of the amino acid; in other systems it either increases (yeast phenylalanine system; Fasiolo et al., 1974) or decreases (*E. coli* isoleucine system; Hustedt & Kula, 1977b) the affinity of the amino acid for the synthetase. However, in the yeast valine system, $tRNA^{Val}$ decreases strongly the affinities of the other small ligands (ATP, AMP, and PP_i) for the synthetase, without suppressing the coupling of their binding sites with that of valine. This indicates that $tRNA^{Val}$ affects mainly the ATP (or AMP and PP_i) sites.

The existence of a unique binding site for ATP (or AMP and PP_i) on the synthetase when $tRNA^{Val}$ is bound indicates that the second ATP site overlaps with the tRNA interacting site. Since valine and magnesium act as effectors to promote the specific interaction of AMP on this site, it likely corresponds to the interacting site of the adenosine end of $tRNA^{Val}$ with the synthetase. This fact might have a great importance for the catalytic functioning of yeast valyl-tRNA synthetase. Since the transfer reaction occurs in the absence of magnesium and since magnesium can bind to tRNA interacting sites, it might be that the binding of tRNA or the saturation of some of its interacting sites with magnesium, together with the binding of valine, promotes the catalytically active interaction of the adenosine end of $tRNA^{Val}$ with valyl-tRNA synthetase.

Properties of the Valyl-tRNA Synthetase·Val~AMP Complex. The active-site titration and the nitrocellulose disk filtration experiments indicate that the valine activation reaction is equilibrated. In the presence of low substrate concentrations and in the absence of pyrophosphatase, only 85% of the enzyme is saturated by adenylate, indicating that under these conditions the concentration of PP_i released is sufficient to reverse partially the valine activation step. The addition, in the incubation mixture, of pyrophosphatase (which suppresses the pyrophosphorolysis of adenylate) or the increase of the substrate concentrations (which increases the rate of

valine activation) displaces the equilibrium toward the adenylate complex (Figure 1 and Table I).

In a complete adenylation system, the complex is less stable in the absence of PP_i than in its presence. Since after separation of the free ligands, the complex is very stable and since free ATP and valine labilize it, it appears that PP_i prevents the substrate-promoted instability of the complex. This indicates that PP_i increases the interaction of Val~AMP with the synthetase and that in the absence of PP_i the two moieties of Val~AMP dissociate alternatively from the binding site. When present, ATP and valine compete for their specific binding site leading to a slow dissociation of the adenylate complex followed by a fast hydrolysis of the liberated Val~AMP. This hydrolysis is favored by increasing the temperature and the magnesium concentration. Since free ATP destabilizes the adenylate complex more than free valine does and since the presence of both ATP and valine labilize strongly the complex, the association of ATP (and not solely the absence of PP_i in the enzyme·adenylate complex) likely promotes this rocking movement of the adenylate and thus increases the dissociation rate of the complex (eq 4)

fast process

$$E{\cdot}AMP{\sim}Val{\cdot}PP_i \rightleftharpoons E{\cdot}AMP{\sim}Val + PP_i$$

slow process

$$E{\cdot}AMP{\sim}Val + ATP \rightarrow E{\cdot}AMP{\sim}Val{\cdot}ATP$$

$$E{\cdot}AMP{\sim}Val{\cdot}ATP + ATP \rightarrow E + AMP{\sim}Val + 2ATP \quad (4)$$

or

$$E{\cdot}AMP{\sim}Val{\cdot}ATP + Val \rightarrow E + AMP{\sim}Val + Val + ATP$$

fast process

$$AMP{\sim}Val \rightarrow AMP + Val$$

This explains why at 25 °C, only 85% of the enzyme was found saturated by Val~AMP even in the presence of a high concentration (1 mM) of free substrates and pyrophosphatase (Table I).

The strong labilizing effect of $tRNA_{ox}^{Val}$ on the enzyme·adenylate complex appears a priori surprising, since Val~AMP is a catalytic intermediate in the $tRNA^{Val}$ charging process (Lagerkvist et al., 1977). Similar behavior was also reported for the yeast phenylalanine system (Baltzinger & Remy, 1977) and was interpreted in the light of the existence of a highly labile covalently bound enzyme·Phe intermediate which would be generated from the enzyme·Phe~AMP complex during the transfer step. It was postulated that $tRNA_{ox}^{Phe}$ promotes the synthesis of this catalytic intermediate without being able to accept further the amino acid and that the $tRNA_{ox}^{Phe}$-promoted hydrolysis of the enzyme·Phe~AMP complex reflects the high lability of this covalent intermediate. The existence of such a reaction would imply that in the valine system the $tRNA_{ox}^{Phe}$-promoted labilization of the enzyme·Val~AMP complex is reversed by an excess of AMP. We did not find such an effect of free AMP (at 1 mM) on the rate of the $tRNA_{ox}^{Val}$-promoted hydrolysis of the enzyme-bound adenylate which indicates that a covalent intermediate is unlikely in the valine system.

Since it was shown that $tRNA_{ox}^{Val}$ modifies the kinetic parameters of the [^{32}P]PP_i–ATP exchange catalyzed by valyl-tRNA synthetase, probably by inducing a conformational change on the adenylation site (Kern & Giegé, 1979), it could be that this allosteric effect either increases the hydrolytic action of the synthetase on Val~AMP or decreases the affinity of Val~AMP for the enzyme. If so, the very high lability of the enzyme·adenylate complex, when the three ligands ($tRNA_{ox}^{Val}$, ATP, and valine) are present together (Table VI), reflects the high rate of exchange between the enzyme-bound adenylate and the free substrates in the presence of $tRNA_{ox}^{Val}$. Since valyl-tRNA synthetase possesses a second binding site for ATP which is common with a tRNA interacting site, it is likely that the ATP- and $tRNA_{ox}^{Val}$-induced lability of the complex results from the occupation of this site, $tRNA_{ox}^{Val}$ being, however, more efficient than ATP in promoting this effect (eq 5)

$$E{\cdot}AMP{\sim}Val{\cdot}ATP \xrightarrow[\text{slow}]{+ATP + Val} E + AMP + Val + ATP$$

$$E{\cdot}AMP{\sim}Val{\cdot}tRNA_{ox}^{Val} \xrightarrow[\text{fast}]{+ATP + Val} E + AMP + Val + tRNA_{ox}^{Val} \quad (5)$$

In addition to its labilizing effect on the adenylate complex, $tRNA_{ox}^{Val}$ also increases the rate of pyrophosphorolysis of the adenylate (Figure 3). This probably results also from the $tRNA_{ox}^{Val}$-induced conformational change of the adenylation site.

The question concerns now whether the $tRNA^{Val}$ effect is specifically induced after its periodate oxidation or if other forms of $tRNA^{Val}$ can also induce it. We know that $tRNA^{Val}$, $tRNA_{ox}^{Val}$, and Val-$tRNA^{Val}$ modify similarly the kinetic properties of the valyl-tRNA synthetase catalyzed [^{32}P]-PP_i–ATP exchange (Kern & Giegé, 1979). This means that all these tRNA forms induce the conformational change on the adenylation site and affect similarly the stability of the enzyme-bound Val~AMP. The effect of the native $tRNA^{Val}$ on the stability of the adenylate complex is difficult to test because of the ability of this tRNA to accept valine. However, the incomplete extent of transfer (~90%) of valine from adenylate to $tRNA^{Val}$ indicates the existence of a slow hydrolytic process of the adenylate besides the fast transfer reaction (eq 6)

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

or

$$\xrightarrow{slow} E{\cdot}tRNA^{Val} + AMP + Val \quad (6)$$

As in all tRNA charging systems studied up to today, the extent of the transfer step varies between 70% and 90% [see the review by Loftfield (1972)]. This mechanism may be general, and the tRNA-promoted lability of the enzyme·adenylate complex would be the prerequisite for this reaction.

The tRNA effect we have described is not solely induced by the cognate tRNA but occurs also in the presence of noncognate species (Table V). It is interesting to notice that the destabilization of the adenylate is the most pronounced in the presence of those noncognate tRNAs which are the most easily misaminoacylated. Since the more or less fast rate of aminoacylation of the tRNAs by valyl-tRNA synthetase likely reflects the more or less adequate positioning of their CCA end near the adenylate in order to accept the valine (Ebel et al., 1973), the above-established correlation suggests that the $tRNA_{ox}$-promoted instability of the enzyme-bound adenylate results from the productive interaction of their CCA end with the synthetase. It appears thus, although the affinities of the noncognate tRNAs for valyl-tRNA synthetase are not significantly different from that of the cognate one (Bonnet & Ebel, 1975), that the noncognate tRNAs associate mainly in a nonproductive fashion with the synthetase (i.e., their CCA end does not interact as efficiently as that of $tRNA^{Val}$). This means that noncognate tRNAs do not induce adequately the conformational change of the adenylation site and agrees with

the fact that $tRNA^{Phe}$ neither affects the equilibrium of the valine activation step (Figure 3) nor modifies the kinetic parameters of the $[^{32}P]PP_i$–ATP exchange (Kern & Giegé, 1979).

The comparative study of the pH effect on the $tRNA^{Val}_{ox}$-promoted instability of the adenylate complex and on the $tRNA^{Val}$ charging leads to the same conclusion: increasing the pH from 6.2 to 8.0 favors the productive interaction of the CCA end of tRNA with the enzyme·adenylate complex. Thus, although acidic pH increases the affinity of $tRNA^{Val}$ for the synthetase (Bonnet & Ebel, 1975), the productive interaction is favored at more alkaline pH. This gives an interpretation of the apparent contradiction of the stronger interaction between tRNAs and synthetases at acidic pH and the better catalytic functioning of the tRNA charging systems at alkaline pH (Kern et al., 1975; Kern & Lapointe, 1979b).

The zinc-promoted labilization of the Val~AMP complex can be related to the zinc-induced tRNA-independent hydrolysis of ATP by yeast phenylalanyl-tRNA synthetase (Igloi et al., 1980). We show that, at least in the yeast valylation system, this effect results from a decrease of the stability of the enzyme-bound adenylate. This effect could be similar to that observed for magnesium, but it would occur with a much higher efficiency (the zinc effect occurs in the micromolar range and the magnesium one in the millimolar range); however, it could also be due to the existence of a specific binding site for zinc on valyl-tRNA synthetase.

Misactivation of Amino Acids. The activation of other amino acids than valine (threonine and analogues of valine) by yeast valyl-tRNA synthetase has already been reported (Igloi et al., 1978; Fersht & Dingwall, 1979). We show that cysteine, isoleucine, and alanine can also be activated by this synthetase, however, with a considerably slower rate than valine and threonine. The noncognate enzyme·adenylates (especially the cysteinyl, isoleucyl, and alanyl species) are less stable than the cognate adenylate. In a complete adenylation mixture containing these last amino acids an equilibrium between the adenylate synthesis and its hydrolysis was rapidly reached even in the presence of pyrophosphatase (Figure 8), and no enzyme-bound adenylates could be isolated. However, due to its greater stability, the enzyme·Thr~AMP complex could be isolated and its properties compared to those of the cognate complex. The various labilizing agents (the substrates, zinc or magnesium ions) did not exhibit specific effects on this wrong complex; thus, they cannot be involved in any proofreading mechanism of the activated threonine. In fact it has been demonstrated that the correction occurs after the transfer of the activated threonine to $tRNA^{Val}$, the Thr-$tRNA^{Val}$ being rapidly deacylated by the valyl-tRNA synthetase (Fersht & Dingwall, 1979). We obtained similar results (D. Kern and J. Gangloff, unpublished data). Although the PP_i–ATP exchange catalyzed by valyl-tRNA synthetase occurs only 2 times faster in the presence of valine than in that of threonine, the K_m of threonine exceeds 140 times that of valine. Consequently, no significant activation of threonine by valyl-tRNA synthetase can occur under physiological conditions where both amino acids are present at similar concentrations.

Physical Properties of Valyl-tRNA Synthetase and Behavior during the Catalytic Process. The lower apparent molecular weight of valyl-tRNA synthetase on sucrose gradients as compared to that obtained by other techniques such as sodium dodecyl sulfate gel electrophoresis (102 000 and 130 000, respectively) indicates that the enzyme is not globular and/or has a low density. The slight increase of the apparent molecular weight of the enzyme·$tRNA^{Val}$ complex (176 000) as compared to that expected from the sum of the apparent molecular weights of the synthetase and the $tRNA^{Val}$ (102 000 + 55 000 = 157 000) is probably a consequence of a conformational change induced by $tRNA^{Val}$ after interaction with the synthetase as demonstrated by neutron scattering (Zaccai et al., 1979) and which results in a contraction of the protein. Finally, the apparent molecular weight of the enzyme·$tRNA^{Val}$ complex does not vary whether the small substrates are present or not. This indicates that the enzyme does not dimerize during the catalytic process, a hypothesis which was proposed after finding, under certain experimental conditions, 2 to 1 enzyme·tRNA complexes in the class of large monomeric synthetases (Österberg et al., 1975). This also shows that the conformational changes on the 1 to 1 complex, which would account for the synergistic effects between the binding of the small ligands, are discrete.

Conclusion: Relation between Ligand Binding and Structural Properties of Yeast Valyl-tRNA Synthetase. There is a coupling between the binding sites of ATP, valine, and the amino acid acceptor end of $tRNA^{Val}$ resulting from conformational changes induced by these ligands on the synthetase. Since valine plays a central role in the productive interaction of ATP and the CCA end of the tRNA with the synthetase, it may be that the binding of the amino acid is a prerequisite for generating either totally or partly the active form of the synthetase. This is, however, not a general rule for all synthetases since the active forms of arginyl-, glutamyl-, and glutaminyl-tRNA synthetases are generated only in the presence of their cognate tRNA [see the reviews by Kisselev & Favorova (1974) and by Söll & Schimmel (1974)].

This work shows that yeast valyl-tRNA synthetase possesses only one catalytic center and is fully active in its monomeric form. It also demonstrates the existence of two ATP sites on the enzyme: one accepting the AMP moiety of ATP and the other one involved in the interaction with tRNA and corresponding probably to the binding site of the 3′-terminal adenosine of tRNA. These two nucleotide binding sites are coupled with the valine binding site, since the amino acid increases in the same way the affinity of AMP for both sites. The question arises whether these sites, belonging to the catalytic center of the enzyme, are equivalent for the binding of both ligands or if, on the contrary, there is an unique specific site for ATP and another one for tRNA. We propose that they are symmetrical with respect to the valine site and can therefore accept either ATP for the synthesis of Val~AMP or the 3′-terminal adenosine of the tRNA for the transfer. This means that the two nucleotide binding sites are intrinsically equivalent, but binding renders them nonequivalent as shown for ATP, since adenylate strongly decreases the affinity of the nucleoside triphosphate for the second nucleotide site. This implies also that the free enzyme or the enzyme·Val complex possesses two sites for the acceptor end of tRNA. Whether this corresponds to the existence of two tRNA sites remains open. Since only one $tRNA^{Val}$ binding site could experimentally be detected, it must be assumed, similar to that shown for ATP, that these two tRNA sites are strongly anticooperative. This anticooperative behavior of the synthetase would be a consequence of a conformational transition upon binding of valine and occupation of one of the nucleotide binding sites. In this scheme the selection of the sites is defined by the first binding of either ATP or the acceptor end of tRNA on one of the two nucleotide sites. This scheme gives a functional importance to the sequence duplications which were found in valyl-tRNA synthetase; the duplicated sequences would define the domains responsible for the symmetrical binding properties

of ATP and $tRNA^{Val}$ on the enzyme. Such a behavior might be general for the large monomeric enzymes of the valyl-tRNA synthetase class, it might, in particular, apply to yeast isoleucyl-tRNA synthetase for which two tRNA binding sites were found (Von der Haar & Cramer, 1978).

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