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# Valyl-tRNA Synthetase from Yellow Lupin Seeds: Hydrolysis of the Enzyme-Bound Noncognate Aminoacyl Adenylate as a Possible Mechanism of Increasing Specificity of the Aminoacyl-tRNA Synthetase†

Hieronim Jakubowski

**ABSTRACT:** Lupin valyl-tRNA synthetase catalyzes the hydrolysis of ATP to AMP and pyrophosphate in the absence of tRNA and in the presence of the following amino acids: cysteine ( $K_M = 22.5$  mM;  $V_{rel} = 1$ ), threonine ( $K_M = 16$  mM;  $V_{rel} = 0.2$ ), alanine ( $K_M = 27$  mM;  $V_{rel} = 0.3$ ), serine ( $K_M = 14$  mM;  $V_{rel} = 0.12$ ), and  $\alpha$ -aminobutyrate ( $K_M = 1.5$  mM;  $V_{rel} = 0.1$ ). This activity is due to hydrolysis of the enzyme-bound noncognate aminoacyl adenylates. The enzyme-bound valyl adenylate is apparently protected by the enzyme and slowly decomposes with a rate constant of  $0.018 \text{ min}^{-1}$ , which is 720 times slower than the rate constant of hydrolysis of valyl-tRNA synthetase-bound cysteinyl adenylate ( $k = 13 \text{ min}^{-1}$ ). The  $K_M$  value for ATP in the ATP pyrophosphohydrolase reaction is  $9 \mu\text{M}$  and does not depend on the nature of the amino acid. This value is one order of magnitude lower than the  $K_M$  value for ATP in the reaction of tRNA aminoacylation with valine ( $K_M = 100 \mu\text{M}$ ). The cysteine-dependent ATP pyrophosphohydrolase activity of valyl-tRNA synthetase in the absence of tRNA exhibits pH optimum between 9.5 and 10.5 in glycine-NaOH buffer, is moderately sensitive to KCl (50% and 60% inhibition at 150 and 300 mM KCl, respectively), is not affected by 0.1–2 mM spermine, and exhibits a temperature dependence with an Arrhenius energy of activation,  $E_a = 64.5 \text{ kJ}$ , which is the same

as that for tRNA aminoacylation with valine. Transfer RNA stimulates the reaction to a degree depending on the nature of amino acid, ATP concentration, pH and kind of buffer used, and KCl and spermine concentrations. Changes of magnesium ion concentration in the range 0.25–10 mM do not affect the stimulation. The degree of stimulation by tRNA of the ATP pyrophosphohydrolase activity also does not depend on temperature. The tRNA apparently acts as an allosteric activator, which binds to the enzyme with  $K_{diss} = 20 \text{ nM}$  and increases  $K_M$  for ATP from 9 to  $100 \mu\text{M}$ . The  $K_M$  for amino acids is either not affected ( $K_M = 28 \text{ mM}$  for alanine and 14 mM for serine) or slightly affected ( $K_M = 9.6 \text{ mM}$  for cysteine and 8.6 mM for threonine) by the presence of tRNA. Transfer RNA devoid of amino acid acceptance by periodate oxidation, albeit able to bind to the enzyme as well as intact tRNA, cannot produce these effects and does not inhibit the ATP pyrophosphohydrolase activity of valyl-tRNA synthetase. Lupin valyl-tRNA synthetase apparently rejects noncognate amino acids at the level of aminoacyl adenylate. The contribution of this pathway of rejection to the overall rejection of noncognate amino acids in the presence of tRNA is calculated to be 25%, 19%, 12%, 2.5% and 2% for cysteine, alanine, serine, threonine, and  $\alpha$ -aminobutyrate, respectively.

The available estimates of error frequencies in protein biosynthesis (Loftfield, 1963; Loftfield & Vanderjagt, 1972; Edelmann & Gallant, 1977) indicate that the precision of amino acid incorporation into proteins is far greater than that which can be expected on theoretical grounds (Pauling, 1958). The energy difference for binding two similar amino acids to an enzyme should only allow for a discrimination that is over two orders of magnitude worse than the error limit of 1 in 3000 found experimentally. Also, the energy difference between similar anticodons and a codon could not account for the observed fidelity of protein biosynthesis.

It is well recognized that aminoacyl-tRNA synthetases activate noncognate amino acids but only the cognate one is finally esterified into tRNA (Igloi et al., 1978, and references cited therein). Most aminoacyl-tRNA synthetases exhibit hydrolytic activity toward aminoacyl-tRNA (Schreier & Schimmel, 1972). This activity has been found to be responsible for correcting errors in amino acid activation (Eldred & Schimmel, 1972; Fersht & Kaethner, 1976; von der Haar & Cramer, 1976; Igloi et al., 1977), although hydrolysis of valyl adenylate by isoleucyl-tRNA synthetase before its

transfer to tRNA has also been suggested as a correcting mechanism (Fersht, 1977).

It has previously been found that valyl-tRNA synthetase acts as an ATP pyrophosphohydrolase<sup>1</sup> in the presence of either threonine or  $\alpha$ -aminobutyrate and that the activity was due to hydrolysis of the enzyme-bound noncognate aminoacyl adenylate (Jakubowski, 1978a). Here those observations are extended to other noncognate amino acids activated by valyl-tRNA synthetase, i.e., cysteine, alanine, and serine. The general features of the noncognate amino acid dependent ATP pyrophosphohydrolase<sup>1</sup> activity of the enzyme are described and compared with those of the reaction in the presence of tRNA. Significance of the ATP pyrophosphohydrolase activity of valyl-tRNA synthetase in the absence of tRNA as the mechanism of rejection of noncognate amino acids by the enzyme is discussed.

## Materials and Methods

Valyl-tRNA synthetase from yellow lupin seeds was prepared as previously described (Jakubowski & Pawelkiewicz,

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<sup>1</sup> The designation "ATP pyrophosphohydrolase" is used in this paper as a name for an amino acid dependent activity of lupin valyl-tRNA synthetases, which converts ATP to AMP and pyrophosphate. This activity is independent of tRNA but is enhanced by tRNA to an extent dependent on amino acid. The mechanisms of the ATP pyrophosphohydrolase activity in the presence of tRNA and in its absence are discussed in the paper.

1975). The enzyme was diluted as needed with 20 mM Hepes<sup>2</sup> buffer (pH 8.0) containing 10 mM 2-mercaptoethanol and 0.3 mg/mL BSA.<sup>2</sup>

Unfractionated tRNA from yellow lupin seeds was prepared according to the procedure of Vanderhoef et al. (1970). A 1 mg/mL solution of the tRNA preparation contained 1.2  $\mu$ M tRNA<sup>Val</sup>.

Radioactively labeled valine and ATP were obtained from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia). Nitrocellulose filters (type BA 85) were purchased from Schleicher and Schüll (Dassel, F.R.G.), poly(ethylenimine)-cellulose (PEI-cellulose) was from Merck (Darmstadt, F.R.G.), inorganic pyrophosphatase and RNase A were from Worthington (Freehold, NJ), ATP was from Reanal (Budapest, Hungary), and amino acids were from Sigma (St. Louis, MO).

The amount of valine impurity in amino acid preparations has been estimated from the dilution of the specific activity of 6.9  $\mu$ M [<sup>14</sup>C]valine incorporated to tRNA in the presence of 50 mM amino acid (Fersht & Dingwall, 1979b). Valine was not detected in the serine preparation, and in the preparations of isoleucine, cysteine, DL-homoserine, DL- $\alpha$ -aminobutyrate, threonine, and alanine, 0.02%, 0.02%, 0.006%, 0.01%, 0.0026%, and 0.001% valine was detected, respectively. The detection limit was 0.0005%.

[<sup>32</sup>P]Pyrophosphate was prepared by pyrolysis of [<sup>32</sup>P]-phosphate at 850 °C for 30 min and subsequent purification of the product by Dowex 1-X8 (Cl<sup>-</sup> form) chromatography (Loftfield & Eigner, 1966).

**Preparation of Periodate-Oxidized tRNA.** Periodate oxidation (Baldwin & Berg, 1966) of lupin tRNA was performed in the dark at 25 °C in a total volume of 180  $\mu$ L of a solution with 100 mM sodium acetate (pH 4.5), 10 mM MgCl<sub>2</sub>, 25 mM NaIO<sub>4</sub>, and 20 mg/mL unfractionated lupin tRNA. After 30 min of reaction, 15  $\mu$ L of glycerol was added to destroy excess NaIO<sub>4</sub>, and the incubation was continued for another 10 min in the dark. Oxidized tRNA was precipitated with 2 volumes of cold ethanol, collected by centrifugation, dissolved, and extensively dialyzed against quartz-distilled water. Valine acceptance of the oxidized tRNA was less than 0.2% of that of untreated tRNA. This preparation, when mixed with intact tRNA in a proportion 1:1, suppresses the rate of aminoacylation of the intact tRNA with valine by 50%, which indicates that oxidized tRNA binds to the enzyme with the same affinity as does intact tRNA.

**Nitrocellulose Filter Assay of the Enzyme-Bound Aminoacyl Adenylates.** The enzyme-bound aminoacyl adenylates are retained on nitrocellulose disks (Jakubowski, 1978a,b). The assay medium (150  $\mu$ L) contained 50 mM Hepes (pH 8.0), 10 mM 2-mercaptoethanol, 10 mM MgCl<sub>2</sub>, 1.7  $\mu$ M [<sup>14</sup>C]ATP (1 pmol = 690 cpm), 40 mM amino acid, 5  $\mu$ g/mL inorganic pyrophosphatase, and 0.17  $\mu$ M valyl-tRNA synthetase. After 10 and 60 min at 25 °C, 60- $\mu$ L aliquots were taken out and applied onto ice-cold buffer-washed nitrocellulose disks (2.4 cm i.d.) and immediately filtered. The disks were washed with three 1-mL aliquots of the buffer [20 mM Hepes (pH 8.0), 5 mM 2-mercaptoethanol, and 10% glycerol] and oven dried, and the adsorbed radioactivity was determined by scintillation counting. Blank values (0.3 pmol), determined in the absence of amino acid, were subtracted from the results.

**ATP-PP<sub>i</sub> Exchange.** The exchange of [<sup>32</sup>P]pyrophosphate into ATP was measured by the adsorption of ATP on SB-2

ion-exchange paper (PP<sub>i</sub> form). [<sup>32</sup>P]Pyrophosphate was eluted from the paper with 0.1 M pyrophosphate (pH 8.0) (Eigner & Loftfield, 1974). The assays were performed at 25 °C in a total volume of 38  $\mu$ L of a solution with 50 mM Hepes (pH 8.0), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 0.2 mg/mL BSA, 1 mM ATP, 1 mM [<sup>32</sup>P]pyrophosphate, varying concentrations of amino acids, and 7–70 nM valyl-tRNA synthetase. The reactions were maintained for 10 min, and after that time 20- $\mu$ L aliquots of the reaction mixtures were spotted onto the origin line of a strip of Reeve Angel (Clifton, NJ) SB-2 ion-exchange paper (PP<sub>i</sub> form). The strip was then fastened with staples to a sheet of Whatman 3MM filter paper. After about 40 min of ascending development of the chromatogram in 0.1 M pyrophosphate (pH 8.0), all unexchanged [<sup>32</sup>P]pyrophosphate moved onto 3MM filter paper and the [<sup>32</sup>P]ATP spots remaining at the origin of the SB-2 paper were cut out, dried, and counted.

**tRNA Aminoacylation.** The rate of aminoacylation of tRNA was determined by the precipitation of [<sup>14</sup>C]Val-tRNA on 3MM filter paper disks with 5% trichloroacetic acid (Mans & Novelli, 1961). The assays were performed at 25 °C in a total volume of 38  $\mu$ L of a solution with 50 mM Hepes (pH 8.0), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 0.2 mM EDTA, 0.2 mg/mL BSA, 4 mg/mL unfractionated lupin tRNA (4.16  $\mu$ M tRNA<sup>Val</sup>), 11–176  $\mu$ M [<sup>14</sup>C]valine, 0.03–0.5 mM ATP, and 1 nM valyl-tRNA synthetase. The reactions were maintained for 5 min, and 20- $\mu$ L aliquots were spotted on 3MM filter paper disks (2.4 cm i.d.), which were then washed with ice-cold 5% trichloroacetic acid (3 times for 15 min) and ethanol (once for 15 min). After the disks were dried, the radioactivity was monitored by scintillation counting with a toluene-based scintillant.

**Amino Acid Dependent ATP Pyrophosphohydrolase.** The ATP pyrophosphohydrolase activity of lupin valyl-tRNA synthetase was measured from the formation of [<sup>14</sup>C]AMP from [<sup>14</sup>C]ATP, [<sup>14</sup>C]AMP being separated from [<sup>14</sup>C]ATP and [<sup>14</sup>C]ADP by thin-layer chromatography on PEI-cellulose (Eigner & Loftfield, 1974). After sample application, the chromatograms were prewashed by ascending chromatography with 75% methanol. This treatment considerably improved the separation of nucleotides achieved upon subsequent ascending chromatography in 2 M formate (Na) (pH 3.4).

Unless otherwise stated, the reactions in a total volume of 23  $\mu$ L were maintained at 25 °C for 10 min; 3- $\mu$ L aliquots of the reaction mixtures were used for [<sup>14</sup>C]AMP determinations. The results are corrected for blanks, which were run in the absence of either enzyme or amino acid.

The [<sup>14</sup>C]ATP preparation used in these studies contained 1.8% [<sup>14</sup>C]AMP and 15% [<sup>14</sup>C]ADP. It has been verified that the level of [<sup>14</sup>C]ADP remained constant throughout the experiments, which rules out the possibility of interference of any nucleotide metabolizing enzymes. Also, in the absence of amino acids, lupin valyl-tRNA synthetase does not hydrolyze [<sup>14</sup>C]ATP to an appreciable extent during 1-h incubation.

## Results

**Activation of Natural Amino Acids by Lupin Valyl-tRNA Synthetase.** Lupin valyl-tRNA synthetase, like its counterparts from yeast (Igloi et al., 1978), *Escherichia coli* (Bergmann et al., 1961; Loftfield & Eigner, 1966), and *Bacillus stearothermophilus* (Fersht & Kaethner, 1976), activates, in addition to valine, several naturally occurring amino acids. The kinetic indexes for the amino acids activated by the lupin enzyme are given in Table I. The reactions with noncognate amino acids are characterized by lower relative rates (com-

<sup>2</sup> Abbreviations used: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; BSA, bovine serum albumin; E, aminoacyl-tRNA synthetase (EC 6.1.1.x); AA, amino acid.

Table I: Kinetic Indexes for Amino Acid Substrates of Lupin Valyl-tRNA Synthetase in ATP-PP<sub>i</sub> Exchange Reaction<sup>a</sup>

amino acid	$K_m$ (mM)	$V$ (%)	rel specificity <sup>e</sup>
valine	0.04	100 <sup>b</sup>	1
threonine	8	48	0.0024
cysteine	6	24	0.0016
DL- $\alpha$ -aminobutyrate <sup>d</sup>	1.3	20	0.0062
serine	6	2.5	0.00017
alanine	28	5.5	0.00008
isoleucine	10 <sup>c</sup>	4.9 <sup>c</sup>	0.00019 <sup>c</sup>
DL-homoserine	3.5 <sup>c</sup>	1.2 <sup>c</sup>	0.00014 <sup>c</sup>

<sup>a</sup> pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 1 mM pyrophosphate, 7–70 nM valyl-tRNA synthetase, 25 °C. <sup>b</sup> 100% = 5460 nM·min<sup>-1</sup> in the presence of 7 nM enzyme. <sup>c</sup> Values were not corrected for 0.02% and 0.006% valine present in isoleucine and DL-homoserine preparations, respectively. <sup>d</sup> Calculated for the L isomer. <sup>e</sup> Relative specificity, defined as  $(k_{cat}/K_m)_{AA} / (k_{cat}/K_m)_{Val^{-1}}$ , has been calculated according to Fersht (1974).

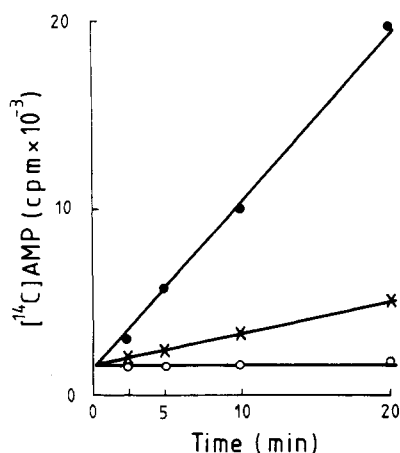


FIGURE 1: Time course of ATP pyrophosphohydrolase activity of lupin valyl-tRNA synthetase in the absence of tRNA and in the presence of cysteine (●), threonine (×), and valine (○). Incubation mixture (30  $\mu$ L) contained 50 mM Hepes (pH 8.0), 10 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mg/mL BSA, 30  $\mu$ M [<sup>14</sup>C]ATP (1 pmol = 690 cpm), 126 nM valyl-tRNA synthetase, and 23.3 mM cysteine, threonine, or valine. The reactions were initiated at 25 °C by the addition of amino acid. After the indicated time intervals, 3- $\mu$ L aliquots were taken out and analyzed for [<sup>14</sup>C]AMP by thin-layer chromatography on PEI-cellulose.

pared with valine) and higher  $K_m$  values for noncognate amino acids than the same reactions catalyzed by the yeast enzyme (Igloi et al., 1978). The relative specificity toward threonine, cysteine, serine, and alanine of the lupin enzyme is, on the average, one order of magnitude higher than that of the yeast enzyme. On the other hand, lupin valyl-tRNA synthetase exhibits almost the same relative specificity toward threonine and alanine as does *E. coli* enzyme (Fersht & Dingwall, 1979b).

**An ATP Pyrophosphohydrolase Activity of Valyl-tRNA Synthetase in the Absence of tRNA.** Lupin valyl-tRNA synthetase hydrolyzes ATP to AMP in the presence of the noncognate amino acids which are activated by the enzyme. The reaction does not require tRNA. Time courses of the tRNA-independent ATP pyrophosphohydrolase activity in the presence of cysteine and threonine are shown in Figure 1. Valine does not support this activity (Figure 1).

In order to exclude that the ATP pyrophosphohydrolase activity might be due to a trace of tRNA impurities in valyl-tRNA synthetase preparation, RNase A was employed. The cysteine-dependent AMP formation in the absence of tRNA was not inhibited by RNase A, which, in turn, suppressed the reaction in the presence of tRNA to the level

Table II: Effect of Time on the Amount of Valyl-tRNA Synthetase-Bound Aminoacyl Adenylates Recovered on Nitrocellulose Disks<sup>a</sup>

amino acid	enzyme-bound aminoacyl [ <sup>14</sup> C]adenylate (pmol/disk)	
	after 10 min	after 60 min
valine	10.0	11.4
threonine	6.9	0.4
cysteine	1.1	0.0
alanine	5.1	0.1
serine	5.4	2.0

<sup>a</sup> pH 8.0, 25 °C, 10 mM MgCl<sub>2</sub>, 40 mM amino acid, 1.7  $\mu$ M [<sup>14</sup>C]ATP, 5  $\mu$ g/mL inorganic pyrophosphatase, 173 nM valyl-tRNA synthetase (10.3 pmol/disk).

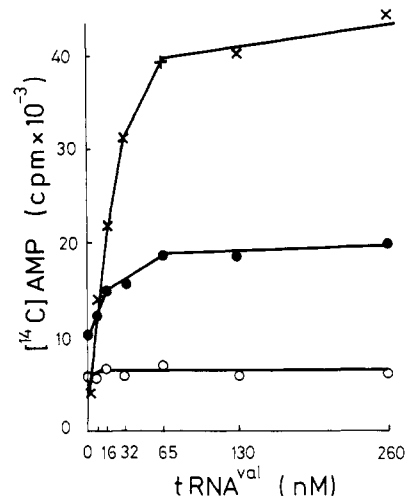


FIGURE 2: Stimulation of the ATP pyrophosphohydrolase activity by tRNA. Assays (total volume 23  $\mu$ L) were carried out in a medium containing 50 mM Hepes (pH 8.0), 10 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mg/mL BSA, 67  $\mu$ M [<sup>14</sup>C]ATP, 18 nM valyl-tRNA synthetase, 34.3 mM amino acid, and unfractionated lupin tRNA (1 mg/mL tRNA = 1.2  $\mu$ M tRNA<sup>Val</sup>) to give the indicated concentrations of tRNA<sup>Val</sup>. The reactions were initiated by the addition of the enzyme and maintained at 25 °C for 20 min. Blanks (without enzyme) were subtracted from the results. The reaction in the presence of alanine (○), cysteine (●), and threonine (×).

observed in the absence of tRNA (not shown).

The tRNA-independent ATP pyrophosphohydrolase activity of valyl-tRNA synthetase is due to hydrolysis of the enzyme-bound noncognate aminoacyl adenylates. The enzyme-bound noncognate aminoacyl adenylates can be isolated by Sephadex gel filtration in very poor yields. When the enzyme-bound aminoacyl adenylate is assayed as a function of time by using nitrocellulose disk filtration, one observes that the amount of valyl adenylate increases slightly with time whereas the amount of noncognate adenylates drops almost to zero after 1 h at 25 °C (Table II). In no case was it possible to obtain the enzyme-bound noncognate adenylate in amounts equal to the amount of valyl adenylate. Two factors contribute to this: (I) greatly diminished rate of adenylate formation with noncognate amino acids (Table I) and (II) considerably diminished stabilities of the enzyme-bound noncognate adenylates (Jakubowski, 1978a, and Table II).

**Stimulation of the ATP Pyrophosphohydrolase Activity by tRNA.** The ATP pyrophosphohydrolase activity of lupin valyl-tRNA synthetase is stimulated by tRNA. The greatest stimulation, 11-fold, has been observed in the presence of threonine. The reactions in the presence of cysteine and alanine have been stimulated 2-fold and 1.2-fold, respectively,

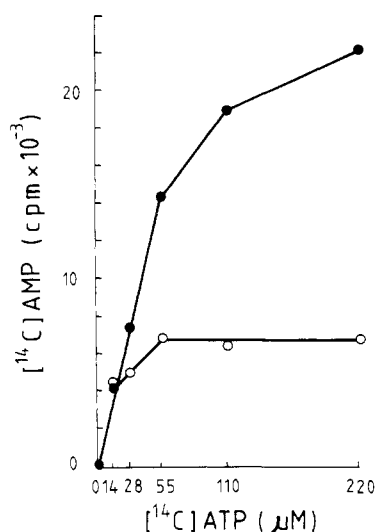


FIGURE 3: Effect of tRNA on the cysteine-dependent ATP pyrophosphohydrolase activity of valyl-tRNA synthetase as a function of ATP concentration. The assays were carried out in a medium containing 50 mM Hepes (pH 8.0), 10 mM  $\text{MgCl}_2$ , 0.2 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mg/mL BSA, 22 mM cysteine, 42 nM valyl-tRNA synthetase, and indicated concentrations of  $[^{14}\text{C}]$ ATP. Reactions in the presence of 0.52  $\mu\text{M}$   $\text{tRNA}^{\text{Val}}$  (●) and in its absence (○).

by saturating concentrations of tRNA. The rate dependence of threonine-, cysteine-, and alanine-dependent ATP pyrophosphohydrolase activity on tRNA concentration is shown in Figure 2. At least the threonine- and cysteine-dependent reactions follow tRNA saturation kinetics, from which a dissociation constant of the enzyme-tRNA complex was calculated to be 20 nM.

The degree of stimulation by tRNA depends on ATP concentration. At ATP concentrations below 15  $\mu\text{M}$ , tRNA has no effect on alanine- (not shown) and cysteine-dependent ATP pyrophosphohydrolase activities, and 3-fold stimulation of the cysteine-dependent reaction is observed at 220  $\mu\text{M}$  ATP (Figure 3). This effect is apparently due to modification of Michaelis constants for ATP and cysteine by tRNA (Table III).

**Kinetic Indexes for the Substrates of Valyl-tRNA Synthetase in ATP Pyrophosphohydrolase Reactions in the Absence of tRNA and in Its Presence.** The kinetic indexes for ATP and amino acids in the ATP pyrophosphohydrolase reactions catalyzed by lupin valyl-tRNA synthetase are given in Table III. It is apparent from the data presented in Table III that tRNA has a dramatic effect on the ATP pyrophosphohydrolase reactions. The tRNA binds to the enzyme with a  $K_{\text{diss}} = 20$  nM (Figure 2) and increases the  $K_M$  value for ATP from 9  $\mu\text{M}$  in the absence of tRNA to 100  $\mu\text{M}$  in its presence. The last value is equal to  $K_M$  for ATP in tRNA aminoacylation with valine (Table III). The  $K_M$  values for threonine and cysteine are about 2-fold reduced by tRNA and those for alanine and serine are not affected.

In the absence of tRNA the cysteine-dependent activity is the highest ( $k = 13 \text{ min}^{-1}$ ) and alanine-, threonine-, serine-, and  $\alpha$ -aminobutyrate-dependent activities are 0.3, 0.2, 0.12, and 0.1 of the cysteine-dependent activity, respectively. In the presence of tRNA, the threonine-dependent activity ( $k = 106 \text{ min}^{-1}$ ) is the highest obtained with noncognate amino acids, and cysteine-, alanine-,  $\alpha$ -aminobutyrate-, and serine-dependent activities are 0.49, 0.20, 0.55, and 0.09 of the threonine-dependent activity, respectively. The rate of the ATP pyrophosphohydrolase activity in the presence of tRNA and valine ( $k = 212 \text{ min}^{-1}$ ) is, within experimental error, equal

Table III: Comparison of the Kinetic Indexes for ATP Pyrophosphohydrolase Activity of Lupin Valyl-tRNA Synthetase in the Presence and Absence of tRNA<sup>a</sup>

substrate	$K_M$ (mM)		$k^f$ ( $\text{min}^{-1}$ )	
	-tRNA	+tRNA <sup>b</sup>	-tRNA	+tRNA <sup>b</sup>
valine		0.033 <sup>c</sup>		195 <sup>c</sup> 212 <sup>e</sup>
threonine	16	7	0.018 <sup>d</sup> 2.7 2.1 <sup>d</sup>	1 <sup>e</sup> 106
DL- $\alpha$ -amino-butyrate	1.5 <sup>h</sup>	2.5 <sup>h</sup>	1.3 0.7 <sup>d</sup>	58
cysteine	22.5	9.6	13	51
serine	14	17	1.5	10
alanine	28	27	4	21
ATP	0.009 <sup>g</sup>	0.1 <sup>c,g</sup>		

<sup>a</sup> pH 8.0, 25 °C, 10 mM  $\text{MgCl}_2$ , 42 nM valyl-tRNA synthetase.

<sup>b</sup> 8.3  $\mu\text{M}$   $\text{tRNA}^{\text{Val}}$ . <sup>c</sup> tRNA aminoacylation, 1 nM valyl-tRNA synthetase. <sup>d</sup> Rate constants of hydrolysis of the enzyme-bound adenylate from Jakubowski (1978a). <sup>e</sup> Rate constant of enzymatic hydrolysis of Val-tRNA calculated from data of Jakubowski & Pawelkiewicz (1977). <sup>f</sup> Calculated by using the  $K_M$  values for ATP shown in the table from the measurements at 0.03–0.06  $\mu\text{M}$   $[^{14}\text{C}]$ ATP. <sup>g</sup> Determined in the presence of cysteine, threonine, and DL- $\alpha$ -aminobutyrate (22 mM each). <sup>h</sup> Calculated for the L isomer.

to the rate of valylation of tRNA and is 2-fold faster than the threonine-dependent ATP pyrophosphohydrolase in the presence of tRNA.

From the data presented in Table III one can conclude that at saturating concentrations of ATP and amino acids the tRNA-independent ATP pyrophosphohydrolase activity of the enzyme comprises 2%, 2.5%, 12%, 19%, and 25% of the tRNA-dependent activity with  $\alpha$ -aminobutyrate, threonine, serine, alanine, and cysteine, respectively. As the tRNA-independent ATP pyrophosphohydrolase activity is not inhibited by tRNA (see below), these figures may represent at least the lower limit of the contribution of hydrolysis of the enzyme-bound noncognate aminoacyl adenylates to the overall rejection of the noncognate amino acids in the presence of tRNA.

**Periodate-Oxidized tRNA Does Not Inhibit the ATP Pyrophosphohydrolase Activity.** It has been shown that the production of AMP during rejection of noncognate amino acids by microbial valyl-tRNA synthetases occurs solely through misacylation of tRNA followed by hydrolysis of misacylated tRNA (Fersht & Kaethner, 1976; Igloi et al., 1977, 1978; Fersht & Dingwall, 1979a,b). Thus, it is essential to show that, with lupin valyl-tRNA synthetase, hydrolysis of the enzyme-bound noncognate aminoacyl adenylates does occur in the presence of tRNA. For this purpose, periodate-oxidized tRNA, which lacks amino acid acceptor activity but still binds to the enzyme, was employed. In contrast with the effect of intact tRNA on the ATP pyrophosphohydrolase activity, periodate-oxidized tRNA only slightly stimulated this activity (Table IV). The threonine-dependent activity was stimulated over 10-fold and less than 2-fold by saturating concentrations of intact tRNA and periodate-oxidized tRNA, respectively. The cysteine-dependent ATP pyrophosphohydrolase activity was stimulated 1.9-fold and 1.3-fold by intact and periodate-oxidized tRNA, respectively. The small stimulatory effect of the periodate-oxidized tRNA indicates that at least a part of the enhanced production of AMP in the presence of intact tRNA might be due to further enhancement of hydrolysis of the enzyme-bound noncognate aminoacyl adenylates. This point, however, requires further experiments. It is interesting to note that the periodate-oxidized tRNA does

Table IV: Comparison of the Effects of Periodate-Oxidized tRNA and Intact tRNA on the ATP Pyrophosphohydrolase Activity of Valyl-tRNA Synthetase<sup>a</sup>

addition (mg/mL)	ATP pyrophosphohydrolase act. (pmol of [ <sup>14</sup> C]AMP/20 min)	
	cysteine dependent	threonine dependent
none	12.4	4.0
oxidized tRNA		
0.016	13.0	5.2
0.032	14.3	6.0
0.065	15.0	6.7
0.130	15.6	7.0
intact tRNA		
0.016	16	22
0.032	20	32
0.065	23	40.5
0.130	23	42

<sup>a</sup> pH 8.0, 25 °C, 10 mM MgCl<sub>2</sub>, 34.3 mM amino acid, 18 nM valyl-tRNA synthetase, and 60 μM [<sup>14</sup>C]ATP.

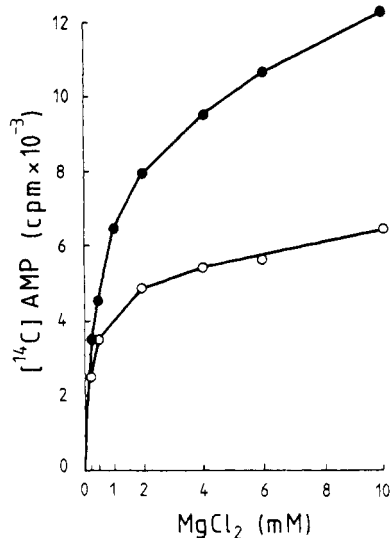


FIGURE 4: Effect of MgCl<sub>2</sub> concentration on the cysteine-dependent ATP pyrophosphohydrolase activity in the presence of 0.11 μM tRNA<sup>Val</sup> (●) and in its absence (○). The assays contained 50 mM Hepes (pH 8.0), 5 mM 2-mercaptoethanol, 0.2 mg/mL BSA, 27 mM cysteine, 57 μM [<sup>14</sup>C]ATP, 38 nM valyl-tRNA synthetase, either 0.11 μM or no tRNA<sup>Val</sup>, and the indicated concentrations of MgCl<sub>2</sub>.

not induce the increase in  $K_M$  for ATP in the ATP pyrophosphohydrolase reaction as the intact tRNA does (not shown).

The data of Table IV indicate that tRNA, when bound to the enzyme, does not inhibit the hydrolysis of the enzyme-bound noncognate aminoacyl adenylates. Thus, it is safe to conclude that the rejection of noncognate amino acids by lupin valyl-tRNA synthetase in the presence of tRNA occurs, at least in part, at the level of the enzyme-bound aminoacyl adenylate.

**Comparison of the tRNA-Independent and tRNA-Dependent ATP Pyrophosphohydrolase Activities of Valyl-tRNA Synthetase in the Presence of Cysteine under Various Conditions.** Both the tRNA-independent and -dependent ATP pyrophosphohydrolase activities of the valine enzyme in the presence of cysteine exhibit similar dependence on magnesium concentrations (Figure 4). The reactions are stimulated by increasing magnesium concentration (up to 10 mM). In the presence of 1 mM Mg<sup>2+</sup>, the reactions proceed at 50–60% of the rates observed in the presence of 10 mM Mg<sup>2+</sup>. Within the range of magnesium concentrations tested, tRNA stimu-

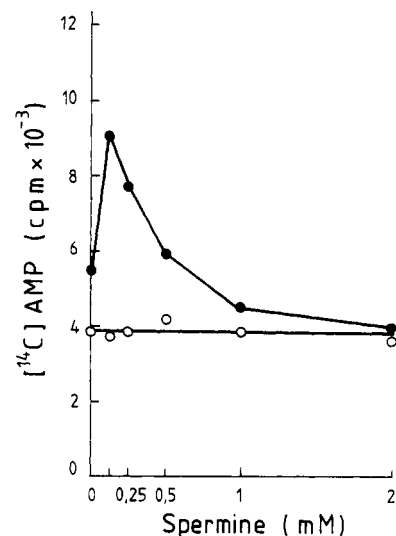


FIGURE 5: Effect of spermine concentration on the cysteine-dependent ATP pyrophosphohydrolase activity in the presence of 0.055 μM tRNA<sup>Val</sup> (●) or in its absence (○). The assays contained 0.25 mM MgCl<sub>2</sub> and indicated concentrations of spermine. For other details see Figure 4.

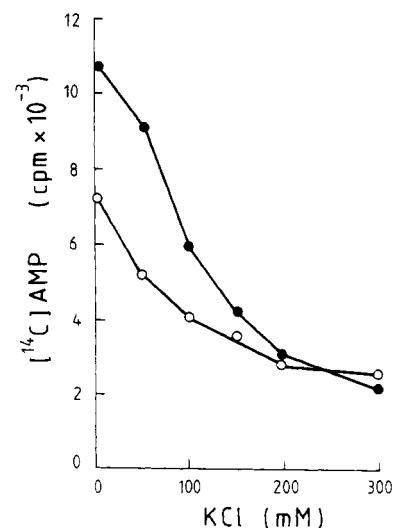


FIGURE 6: Effect of KCl concentration on the cysteine-dependent ATP pyrophosphohydrolase activity. The assays were carried out under the conditions of Figure 4, except MgCl<sub>2</sub> concentration was 10 mM and indicated concentrations of KCl were included. Reactions in the presence of 0.055 μM tRNA<sup>Val</sup> (●) and in its absence (○).

lates the enzyme from 1.4-fold in the presence of 0.25 mM Mg<sup>2+</sup> to 1.9-fold in the presence of 10 mM Mg<sup>2+</sup>.

The tRNA-independent ATP pyrophosphohydrolase activity does not depend on spermine (up to 2 mM). Spermine, however, affects the rate of the tRNA-dependent reaction (Figure 5). Stimulation is observed in the presence of 0.12 mM spermine followed by a gradual inhibition when the concentration of spermine increases. The tRNA-dependent activity is depressed by 2 mM spermine to the level of the tRNA-independent activity. This experiment supports the contention that tRNA is not absolutely required for the ATP pyrophosphohydrolase activity to occur.

Both tRNA-independent and -dependent ATP pyrophosphohydrolase activities of valyl-tRNA synthetase are inhibited by KCl, albeit to a different extent (Figure 6). The tRNA-dependent activity is more sensitive to KCl inhibition than the tRNA-independent activity. Both activities converge in the presence of 0.2–0.3 M KCl, and 70% and 80% inhibition

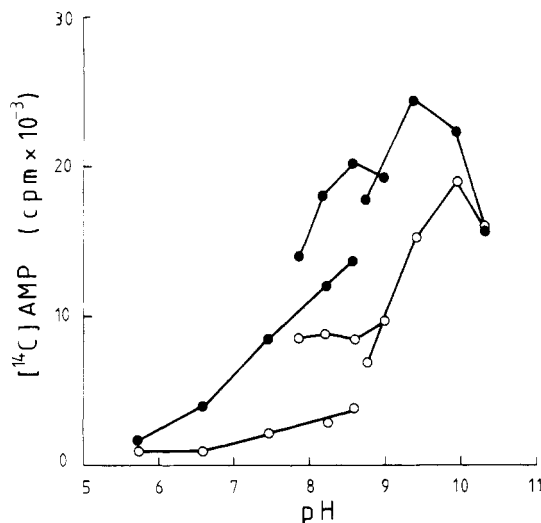


FIGURE 7: Effect of pH on the cysteine-dependent ATP pyrophosphohydrolase activity. The assays contained buffer or indicated pH, 5 mM 2-mercaptoethanol, 10 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.2 mg/mL BSA, 21 mM cysteine, 34  $\mu$ M [<sup>14</sup>C]ATP, 38 nM valyl-tRNA synthetase, and either 0.057  $\mu$ M tRNA<sup>Val</sup> (●) or no tRNA<sup>Val</sup> (○). The following buffers were used: 25 mM potassium phosphate (pH 5.75–8.6), 50 mM Hepes–KOH (pH 7.85–9.0), and 50 mM glycine–NaOH (pH 8.75–10.35). The reactions were maintained at 25 °C for 20 min.

of the tRNA-independent and -dependent activities, respectively, is observed. It should be noted that 0.3 M KCl completely abolishes the aminoacylation of tRNA catalyzed by valyl-tRNA synthetase (Jakubowski & Pawelkiewicz, 1977), most likely by removing tRNA from the enzyme as has been shown for *E. coli* valyl-tRNA synthetase (Loftfield & Eigner, 1967). Thus, at high salt concentrations the tRNA-dependent activity should become tRNA independent, which is actually seen in Figure 6. Again, the experiment shown in Figure 6 argues against the presence of a trace of tRNA impurities in valyl-tRNA synthetase preparation, which could be responsible for the apparent ATP pyrophosphohydrolase activity of the enzyme.

The ATP pyrophosphohydrolase activities of the valine enzyme have been assayed in various buffers in the pH range from 5.75 to 10.4. The rate of the tRNA-independent reaction increases with increasing pH and exhibits an optimum at pH 10.0 (Figure 7). tRNA stimulates the reaction to an extent, depending on pH and kind of buffer used. The highest effect of tRNA has been observed in phosphate buffers (4-fold stimulation regardless of pH within the pH range 6.5–8.5). A 1.5- to 2-fold stimulation has been observed in Hepes buffers of pH 7.8–9.0. The stimulation decreased from 2.5- to 1-fold in glycine–NaOH buffers of pH 8.75–10.4.

The ATP pyrophosphohydrolase activity increases almost 10-fold as temperature increases from 10 to 35 °C. An Arrhenius energy of activation of 64.5 kJ can be calculated from the data (Figure 8). Aminoacylation of tRNA with valine exhibits the same energy of activation (not shown). tRNA stimulates the ATP pyrophosphohydrolase reaction 1.5-fold regardless of temperature.

#### Discussion

The purpose of the investigation described in this paper was to characterize the reaction of hydrolysis of the enzyme-bound noncognate aminoacyl adenylates and to determine what is the contribution of this reaction to overall rejection of noncognate amino acids by lupin valyl-tRNA synthetase. It has

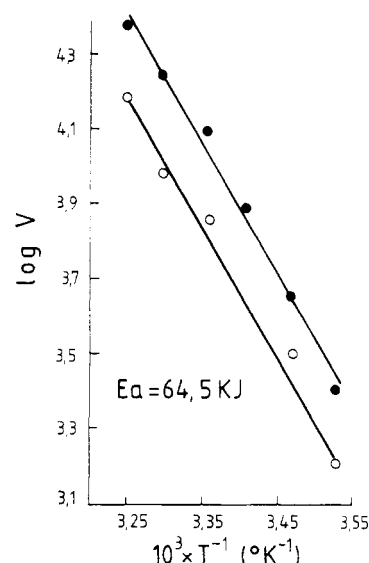
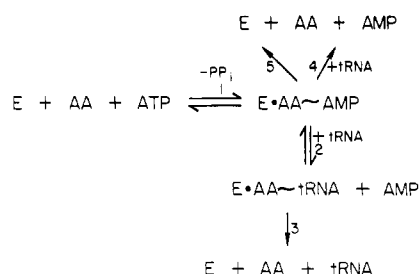


FIGURE 8: Effect of temperature on the cysteine-dependent ATP pyrophosphohydrolase reaction in the presence of 0.057  $\mu$ M tRNA<sup>Val</sup> (●) and in its absence (○) (Arrhenius plot). The assays were carried out at the indicated temperatures under the conditions described in the legend to Figure 7, except 50 mM Hepes (pH 8.0) was used as a buffer, cysteine concentration was 27 mM, and the reactions were maintained at 25 °C for 10 min.

been previously shown that  $\alpha$ -aminobutyryl (half-life 1 min) and threonyl (half-life 0.3 min) adenylates formed with valyl-tRNA synthetase are 40 and 120 times less stable, respectively, than the enzyme-bound valyl adenylate [half-life 40 min; Jakubowski (1978a)]. Here this observation has been extended to several other noncognate aminoacyl adenylates formed with cysteine, alanine, and serine (Table II). Incubation of the enzyme, ATP, and either cysteine, alanine, threonine, serine, or  $\alpha$ -aminobutyrate leads to hydrolysis of ATP to AMP, which is due to rapid formation (Table I) and subsequent slow hydrolysis of the enzyme-bound noncognate aminoacyl adenylates (Table III). For example, the enzyme-bound cysteinyl adenylate forms with a rate constant  $k = 187 \text{ min}^{-1}$  (Table I) and hydrolyzes with a rate constant  $k = 13 \text{ min}^{-1}$  (Table III). The rate constants of formation of the enzyme-bound valyl adenylate and its hydrolysis are  $780 \text{ min}^{-1}$  and  $0.018 \text{ min}^{-1}$ , respectively. Thus, the enzyme-bound cysteinyl adenylate is 720 times less stable than the enzyme-bound valyl adenylate. The stabilities of the enzyme-bound alanyl and seryl adenylates are 220 and 83 times lower, respectively, than the stability of the enzyme-bound valyl adenylate (Table III). The hydrolysis of the enzyme-bound noncognate aminoacyl adenylates exhibits properties compatible with kinetic proofreading (Hopfield, 1974).

The possible contribution of the hydrolysis of the enzyme-bound noncognate aminoacyl adenylates to the overall rejection of noncognate amino acids by lupin valyl-tRNA synthetase has been assessed by comparing the ATP pyrophosphohydrolase reactions catalyzed by the enzyme in the absence and presence of tRNA. The tRNA-independent ATP pyrophosphohydrolase activity was stimulated by tRNA ( $K_{\text{diss}} = 20 \text{ nM}$  for tRNA<sup>Val</sup>) to an extent different for various amino acids (Figure 2). The stimulation was apparently dependent on ATP concentration (Figure 3), and it was also modified by changes of pH (Figure 7), salt (Figure 6), and spermine (Figure 5) concentrations. Magnesium (Figure 4) and temperature (Figure 8) have only negligibly small (if any) effects on the stimulation of the ATP pyrophosphohydrolase activity by tRNA. The data presented in Figures 2–8 indicate that

Scheme I



the ATP pyrophosphohydrolase activity in the absence of tRNA may constitute up to 100% of the activity in the presence of tRNA, at least during rejection of cysteine by valyl-tRNA synthetase.

Due to the very low dissociation constant of the enzyme-tRNA complex ( $K_{\text{diss}} = 20$  nM, Figure 2) and high enzyme concentrations used in these studies, one can argue that the ATP pyrophosphohydrolase activity observed in the absence of exogenously added tRNA might in fact be caused by a trace of tRNA impurities present in the enzyme preparation. However, several pieces of evidence indicate that this is unlikely. First, the ATP pyrophosphohydrolase activity in the absence of tRNA is not affected by RNase, whereas the reaction in the presence of tRNA is suppressed by RNase to the level observed in the absence of tRNA. Second, this activity persists under the conditions which lead to complete dissociation of tRNA from the enzyme, e.g., at high pH values or in the presence of high salt concentrations (Loftfield & Eigner, 1967; Lam & Schimmel, 1975). Third, the apparent ATP pyrophosphohydrolase activity is not inhibited by periodate oxidized tRNA, which still is able to bind to the enzyme (Table IV).

The rejection pathways used by aminoacyl-tRNA synthetases can be described by Scheme I. In reaction 1, amino acid is activated to form enzyme-bound aminoacyl adenylate. The discrimination against noncognate amino acid, mostly accomplished by preferential binding of cognate amino acid, is between  $10^{-2}$  and  $10^{-4}$  (e.g., see Table I). The enzyme-bound aminoacyl adenylate then either reacts with tRNA (reaction 2) or hydrolyzes in a tRNA-dependent (reaction 4) or tRNA-independent (reaction 5) reaction. Reaction 2 has been shown to occur during rejection of threonine and  $\alpha$ -aminobutyrate by *B. stearothermophilus* and yeast valyl-tRNA synthetases (Fersht & Kaethner, 1976; Fersht & Dingwall, 1979a), and it has also been observed with modified (devoted of hydrolytic function) yeast tRNAs specific for isoleucine (von der Haar & Cramer, 1976), valine, and phenylalanine (Igloi et al., 1977, 1978). No discrimination in this reaction has been reported, but, if we assume that most of the ATP pyrophosphohydrolase activity of lupin valyl-tRNA synthetase is due to reactions 2 and 3, the lupin enzyme could discriminate against noncognate amino acids in reaction 2. The rate constants for this reaction with noncognate amino acids are from 20-fold (with serine) to 2-fold (with threonine) lower compared with valine (Table III). Reaction 3 has been originally described for *E. coli* isoleucyl-tRNA synthetase (Schreier & Schimmel, 1972), and it was suggested to be involved in rejection of misactivated valine by the enzyme (Eldred & Schimmel, 1972). This reaction is  $10^2$ – $10^3$  times faster with noncognate aminoacyl-tRNAs than with cognate ones and is the main rejection pathway used by valyl-tRNA synthetases from yeast (Igloi et al., 1977, 1978) and *B. stearothermophilus* and *E. coli* (Fersht & Kaethner, 1976; Fersht & Dingwall, 1979a). Lupin valyl-tRNA synthetase catalyzes reaction 3

with Val-tRNA ( $k = 1 \text{ min}^{-1}$ ; Table III). Reaction 4 has been suggested to occur during rejection of valine by *E. coli* isoleucyl-tRNA synthetase (Fersht, 1977).

Reaction 5 is negligibly slow ( $k = 0.04 \text{ min}^{-1}$ ) with both enzyme-bound valyl and isoleucyl adenylates compared with reaction 3 ( $k = 72 \text{ min}^{-1}$ ) for isoleucyl-tRNA synthetase (Fersht, 1977). There is an indication that reaction 5 might occur during rejection of homocysteine by *B. stearothermophilus* methionyl-tRNA synthetase (Fersht & Dingwall, 1979c). Reaction 5 is very slow with lupin valyl-tRNA synthetase-bound valyl adenylate ( $k = 0.018 \text{ min}^{-1}$ ), the adenylate being apparently protected by the enzyme against hydrolysis (Fersht, 1977), and it becomes significant with the enzyme-bound noncognate aminoacyl adenylates formed with cysteine ( $k = 13 \text{ min}^{-1}$ ), alanine ( $k = 4 \text{ min}^{-1}$ ), threonine ( $k = 2.7 \text{ min}^{-1}$ ),  $\alpha$ -aminobutyrate ( $k = 1.3 \text{ min}^{-1}$ ), and serine ( $k = 1.5 \text{ min}^{-1}$ ) (Table III). This reaction may constitute as much as 25% of the reactions leading finally to hydrolysis of noncognate adenylates formed with lupin valyl-tRNA synthetase and may be an additional or alternate rejection pathway for the enzyme. At least with cysteine the hydrolysis of the enzyme-bound noncognate aminoacyl adenylate ( $k = 13 \text{ min}^{-1}$ ) is considerably faster than the enzymatic hydrolysis of Val-tRNA ( $k = 1 \text{ min}^{-1}$ ) (Table III). The contribution of reaction 5 to overall rejection of noncognate amino acids may vary with experimental conditions. In particular, in the presence of low concentrations of ATP (Figure 3), the rate of reaction 5 may be equal to that of reaction 2.

Finally, a question which remains to be answered is to what extent and by which pathway does lupin valyl-tRNA synthetase reject noncognate amino acids in vivo. Despite rather great initial discrimination against noncognate amino acids (see Table I), the enzyme is encountered with difficult specificity problems in vivo. Threonine, cysteine, serine, and alanine are present in the plant at concentrations 30-, 12-, 97-, and 20-fold greater, respectively, than that of valine (Nowacki & Waller, 1973). When the relationship described by Fersht (1974) and the data of Table I are used, it can be calculated (simply by multiplying the values of relative specificity from Table I for a given amino acid by the fold molar excess of that amino acid over valine) that 72 mol of threonine, 19 mol of cysteine, 16 mol of serine, and 1.6 mol of alanine may be activated per 1000 mol of valine, which makes roughly 1 mol of the enzyme-bound noncognate aminoacyl adenylate per 10 mol of the enzyme-bound valyl adenylate formed in vivo. A competition between noncognate amino acid and valine determined experimentally (not shown) is compatible with the kinetic constants for cognate and noncognate reactions (Tables I and III). It may be calculated that out of the 10% errors made by valyl-tRNA synthetase in vivo, about one-tenth may be rejected at the level of aminoacyl adenylate, provided that ATP and tRNA are present at saturating concentrations. The contribution of the hydrolysis of the enzyme-bound noncognate aminoacyl adenylate to overall rejection of noncognate amino acids is greater at limiting concentrations of ATP (Figure 3) and tRNA (Figure 2). At least in one physiological state, i.e., during the first hours of germination, the concentrations of ATP in lupin tissue are very low (10–100  $\mu\text{M}$ ; Dzięgielewska et al., 1979) and the availability of intact tRNA is limited (Dzięgielewska & Pawełkiewicz, 1977). Thus, it is likely that during that physiological state, the hydrolysis of the enzyme-bound noncognate aminoacyl adenylates may be an important pathway of rejection of noncognate amino acids used by lupin valyl-tRNA synthetase.



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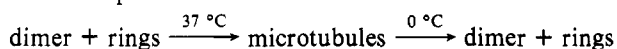
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## Exchange of Tubulin Dimer into Rings in Microtubule Assembly-Disassembly<sup>†</sup>

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**ABSTRACT:** We have prepared native radioactive tubulin dimer from two species: [<sup>35</sup>S]tubulin dimer, by in vivo labeling of rat brain, and porcine [<sup>3</sup>H]ethyltubulin, as previously described [Zeeberg, B., Cheek, J., & Caplow, M. (1980) *Anal. Biochem.* 104, 321-327]. After microtubule assembly with radioactive tubulin dimer and nonradioactive dimer and rings, the tubulin in the rings and the dimer obtained upon disassembly have approximately equal specific activities. Therefore, during the reaction sequence



the tubulin initially in rings becomes indistinguishable from tubulin initially in dimer. Under nonpolymerizing conditions (0 °C) radioactive tubulin dimer and radioactive guanine

nucleotide are incorporated into rings at approximately equal rates. This indicates that there is a pathway for nucleotide incorporation into rings under nonpolymerizing conditions which involves the incorporation of dimer-bound nucleotide. We also report results on the lack of the mirror image equilibrium during the disassembly process, using porcine [<sup>3</sup>H]-ethyltubulin dimer, rat [<sup>35</sup>S]tubulin dimer, and a [<sup>3</sup>H]-GDP-porcine tubulin dimer complex. In all three cases there is no significant disassembly-dependent incorporation of radioactivity into rings when microtubules are disassembled in the presence of radioactive dimer. These results demonstrate that, for rat and porcine tubulin, rings are formed during microtubule disassembly by direct cleavage of intact rings, without a tubulin dimer intermediate.

**M**icrotubules have been postulated to be in a dynamic equilibrium with tubulin subunits in the cell (Inoue & Sato,

1967). The central role of microtubules in the mitotic apparatus and cytoskeleton has led investigators to examine the regulatory elements for in vivo microtubule properties and functioning. A number of recent studies have concentrated on the mechanism for microtubule assembly and disassembly (Margolis & Wilson, 1978, 1979; Karr & Purich, 1979; Sternlicht & Ringel, 1979; Bergen & Borisy, 1980).

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