Demaille, J. G., Peters, K. A., & Fischer, E. H. (1977) *Biochemistry 16*, 3080.
Erickson, B. W., & Merrifield, R. B. (1976) *Proteins 2*, 255.
Gold, M. H., & Segel, I. H. (1974) *J. Biol. Chem. 249*, 2417.
Gupta, R. K., Oesterling, M. M., & Mildvan, A. S. (1976) *Biochemistry 15*, 2881.
Hartley, B. S. (1970) *Biochem. J. 119*, 805.
Huiging, F., & Larner, J. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 647.
Jallon, J. M., & Cohn, M. (1970) *Biochem. Biophys. Acta 222*, 542.
Kemp, B. E., Graves, D. J., Benjamine, E., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 4888.
Krebs, E. G. (1972) *Curr. Top. Cell. Regul. 5*, 99.
Li, T. M., Mildvan, A. S., & Switzer, R. L. (1978) *J. Biol. Chem. 253*, 3918.
Maller, J. L., Kemp, B. E., & Krebs, E. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 248.
Merritt, E. A., Sundaralingam, M., Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3274.
Mildvan, A. S. (1970) *Enzymes, 3rd Ed. 2*, 445.
Mildvan, A. S., & Cohn, M. (1963) *Biochemistry 2*, 910.
Mildvan, A. S., & Cohn, M. (1966) *J. Biol. Chem. 241*, 1178.
Mildvan, A. S., & Engle, J. L. (1972) *Methods Enzymol. 26C*, 654.
Milner-White, E. J., & Watts, D. C. (1971) *Biochem. J. 122*, 727.
Miyamoto, E., Kuo, J. F., & Greengard, P. (1969) *J. Biol. Chem. 244*, 6395.
Miziorko, H. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 2743.
Moll, G. W., Jr., & Kaiser, E. T. (1976) *J. Biol. Chem. 251*, 3993.
Moll, G. M., Jr., & Kaiser, E. T. (1977) *J. Biol. Chem. 252*, 3007.
O'Sullivan, W. J., & Cohn, M. (1966) *J. Biol. Chem. 241*, 3104.
Pomerantz, A. H., Allfrey, V. G., Merrifield, R. B., & Johnson, E. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4261.
Reed, G. H., & Cohn, M., (1972) *J. Biol. Chem. 247*, 3073.
Stewart, J. M., & Young, J. D. (1968) *Solid Phase Peptide Synthesis*, W. H. Freeman, San Francisco, CA.
Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J. 159*, 409.
Walsh, D. A., & Krebs, E. G. (1973) *Enzymes, 3rd Ed. 8*, 555.
Witt, J. J., & Roskoski, R., Jr. (1975) *Anal. Biochem. 66*, 243.
Yount, R. G., Babcock, D., Ojala, D., & Ballantyne, W. (1971) *Biochemistry 10*, 2484.
Zetterquist, O., Ragnarsson, U., Humble, E., Berglund, L., & Engstrom, L. (1976) *Biochem. Biophys. Res. Commun. 70*, 696.

Establishing the Misacylation/Deacylation of the tRNA Pathway for the Editing Mechanism of Prokaryotic and Eukaryotic Valyl-tRNA Synthetases[+]

Alan R. Fersht* and Colin Dingwall

ABSTRACT: The valyl-tRNA synthetases from *Bacillus stearothermophilus* and *Escherichia coli* have been shown to edit the mistaken activation of L-α-aminobutyrate (αBut) by first misacylating $tRNA^{Val}$ and then specifically deacylating the αBut-$tRNA^{Val}$. This pathway was established initially by rapid quenching and sampling experiments which detected the transiently formed αBut-$tRNA^{Val}$ on the addition of $tRNA^{Val}$ to the preformed and isolated E·αBut-AMP complex. The misacylation/deacylation pathway of editing was then shown to occur in the normal reaction in the steady state by isolation of the mischarged tRNA. For example, [^{14}C]-αBut-$tRNA^{Val}$ (*E. coli*) can be isolated from a steady-state reaction mixture containing [^{14}C]-αBut (0.9 mM), enzyme (71 μM), $tRNA^{Val}$ (72 μM), and ATP (8 mM), at the steady-state concentration predicted from the measured deacylation rate constant (50 s^{-1}) and the steady-state kinetic constants for the ATP/pyrophosphatase activity under these conditions. The same pathway appears to operate for the rejection of threonine by the eukaryotic valyl-tRNA synthetase since, on mixing the enzyme-bound threonyl adenylate and tRNA from yeast, transiently formed Thr-$tRNA^{Val}$ is observed and its deacylation rate constant estimated to be greater than 80 s^{-1}. The rate-determining step of the valyl-tRNA synthetase catalyzed deacylation of αBut-$tRNA^{Val}$ is shown to be different in nature as well as in magnitude from that of Val-$tRNA^{Val}$. The rate of the editing reaction is independent of pH between 6 and 7.8 and is unaffected by the addition of aminoacyl adenylate, whilst the rate of deacylation of Val-$tRNA^{Val}$ follows an ionization of pK_a = 8 and is decreased by the addition of aminoacyl adenylate. A unified scheme is presented that could account for the specificity in the deacylation reaction.

The fidelity of protein biosynthesis is far higher than expected from simple kinetic and thermodynamic arguments. One link in the synthetic chain where errors are likely to occur is at the aminoacylation of tRNA, for it is here that the cell has to distinguish between amino acids that are structurally very similar. The discrimination is accomplished by: (a) the aminoacyl-tRNA synthetases discriminating as precisely as possible between competing amino acids by the preferential binding of the cognate amino acid; (b) where necessary, the enzymes having evolved an *editing* function whereby mistakenly aminoacylated intermediates or products are hy-

[+] From the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, England. *Received June 13, 1978; revised manuscript received December 11, 1978.*
* Address from Sept 1978 to Sept 1979: Department of Biochemistry, Stanford University Medical Center, Stanford, CA 94305.

0006-2960/79/0418-1238$01.00/0 © 1979 American Chemical Society

drolytically destroyed. The editing activity was discovered by (Norris) Baldwin & Berg (1965, 1966). The isoleucyl-tRNA synthetase of *E. coli* activates valine to form an isolable enzme-bound valyl adenylate complex. But whereas the addition of $tRNA^{Ile}$ to the enzyme-bound isoleucyl adenylate complex leads to the formation of Ile-$tRNA^{Ile}$, the addition to the valyl adenylate complex leads to its quantitative hydrolysis. Thus, in the presence of valine and $tRNA^{Ile}$, the isoleucyl-tRNA synthetase acts as an ATP/pyrophosphatase, wastefully but necessarily hydrolyzing ATP to AMP as the valyl adenylate turns over (eq 1). Without the editing

$$\text{IRS} \xrightarrow[\text{PP}]{\text{ATP,Val}} \text{IRS·Val-AMP} \xrightarrow{tRNA^{Ile}} \text{IRS + Val + AMP} \quad (1)$$

mechanism, the error rate for the misincorporation of valine for isoleucine would be about 1 in 16 (Mulvey & Fersht, 1977b) and would be expected to lead to amino acid substitutions in surviving completed proteins at a rate much higher than the value of 1/3000 found in vivo for the rabbit reticulocyte globin (Loftfield & Vanderjagt, 1972).

One crucial question on the editing mechanism is whether the binding of tRNA induces the direct hydrolysis of the aminoacyl adenylate or the amino acid is transferred to the tRNA and the misacylated tRNA deacylated. In support of the latter, it has been found that aminoacyl-tRNA synthetases have a hydrolytic activity toward aminoacylated tRNA (Yarus, 1972; Eldred & Schimmel, 1972). In most cases, such as for the hydrolysis of a correctly charged cognate tRNA, the activity is extremely weak. But, in two examples, the hydrolysis of Val-$tRNA^{Ile}$ catalyzed by the isoleucyl-tRNA synthetase and Val-$tRNA^{Phe}$ by the phenylalanyl-tRNA synthetase, the values of k_{cat} are higher, being about 0.02 s^{-1} for the former at 3 °C and about 2 s^{-1} for the latter at 37 °C (Yarus, 1972; Eldred & Schimmel, 1972). It was thus suggested that editing occurred by misacylation followed by corrective deacylation. This hypothesis was subsequently criticized because the two reported hydrolytic rates are too low to have any substantial effect on error correction (Bonnet & Ebel, 1974). Also, recent evidence on a wider range of deacylation reactions suggests that, in general, they are not sufficiently preferential for mischarged tRNA and are caused by a nonspecific activation of the ester bond on binding the aminoacyl-tRNA to the enzyme (Bonnet, 1974; Sourgoutchoff et al., 1974).

We have recently provided the first direct evidence that some editing does take place by misacylation followed by deacylation. Using a rapid quenching apparatus that enabled us to sample reaction mixtures from 5 ms after mixing reagents, we were able to trap the transiently mischarged threonyl-$tRNA^{Val}$ during the rejection of threonine by the valyl-tRNA synthetase from *Bacillus stearothermophilus* (Fersht & Kaethner, 1976). The enzyme-catalyzed deacylation of the Thr-$tRNA^{Val}$ has the high turnover number of 40 s^{-1} at 25 °C. However, in a subsequent study on the rejection of valine by the isoleucyl-tRNA synthetase from *Escherichia coli* (Fersht, 1977a), little transiently mischarged Val-$tRNA^{Ile}$ could be detected, although accumulation was predicted from the measured turnover number of 10 s^{-1} at 25 °C for the enzyme-catalyzed deacylation. This result is inconclusive. Either the measurement of the turnover number for deacylation is artefactually low or editing occurs at the level of Val-AMP hydrolysis.

Other experimental evidence is ambiguous. From the observation that 3′-deoxy-$tRNA^{Ile}$ from yeast may be successfully mischarged with valine (von der Haar & Cramer, 1976) and 3′-deoxy-$tRNA^{Val}$ may be mischarged to some extent with threonine (Igloi et al., 1977), it was proposed that the yeast enzymes edit by the deacylation of mischarged tRNA. But, just because the 3′-deoxy-tRNAs may be misacylated does not necessarily imply that the normal tRNAs may also be misacylated. In the absence of direct trapping experiments, it may equally well be argued that the 3′-OH group of the tRNA is involved in destroying the enzme-bound aminoacyl adenylate before the formation of mischarged aminoacyl-tRNA. Also, the reported turnover numbers for the isoleucyl-tRNA synthetase (yeast) catalyzed deacylation of synthetically mischarged Val-$tRNA^{Ile}$ (0.16 s^{-1} at 36 °C, von der Haar & Cramer, 1976) and the valyl-tRNA synthetase (yeast) catalyzed deacylation of Thr-$tRNA^{Val}$ (0.5 s^{-1} at 37 °C, Igloi et al., 1977) are orders of magnitude too low to support the proposed pathway (Fersht, 1977a; Mulvey & Fersht, 1977b) and at face value would appear to be consistent with editing *before* the transfer of amino acid to tRNA.

In this study, we attempt to generalize the misacylation/deacylation pathway by extending our rapid quenching measurements to the rejection of α-aminobutyrate (αBut)[1] by the valyl-tRNA synthetases from *E. coli* and *B. stearothermophilus* and of threonine by the enzyme from yeast. We also examine the enzyme-catalyzed deacylation of Val-$tRNA^{Val}$ and αBut-$tRNA^{Val}$ to compare the "nonspecific" and "editing" hydrolyses. The use of the unnatural amino acid has the advantage that there are no complications caused by the presence of an α-aminobutyryl-tRNA synthetase and $tRNA^{\alpha But}$. This has facilitated a search for the accumulation of αBut-$tRNA^{Val}$ under steady-state conditions.

Experimental Section

Materials. The valyl-tRNA synthetases from *B. stearothermophilus* and *E. coli* were obtained as described previously (Fersht & Kaethner, 1976; Mulvey & Fersht, 1977a). The valyl-tRNA synthetase from yeast was a generous gift from Dr. R. Giegé. All enzymes were purified to homogeneity. Each preparation ran as a single band on polyacrylamide gel electrophoresis with sodium dodecyl sulfate. Active site titration (Fersht et al., 1975) gave a purity of 96% for the bacterial enzymes and 83% for the yeast. The concentrations of the enzyme solutions were calculated from the molar absorbances and molecular weights using the previously reported values for the bacterial enzymes (Mulvey & Fersht, 1977a) and A_{280} = 1.79 cm^{-1} mg^{-1} mL^{-1} and mol wt = 130 000 for the yeast enzyme (R. Giegé, personal communication). The tRNA from *B. stearothermophilus* (valine acceptance = 1400 pmol/A_{260}) and from *E. coli* (valine acceptance = 460 pmol/A_{260}) have been described previously (Fersht & Kaethner, 1976; Mulvey & Fersht, 1977b). Unfractionated yeast tRNA (valine acceptance = 120 pmol/A_{260}) was obtained from Boehringer. ^{14}C-labeled valine (specific activity = 270 Ci/mol), ^{14}C-labeled threonine (232 Ci/mol), and potassium [^{14}C]cyanide (57.8 Ci/mol) were obtained from the Radiochemical Centre, Amersham, England. α-L-Aminobutyrate was obtained either from Sigma or Fluka and recrystallized before use from ethanol/water. Propionaldehyde was obtained from Aldrich and twice fractionally distilled immediately before use. Hog kidney acylase I was obtained from Sigma.

[1] Abbreviations used: αBut, α-L-aminobutyric acid; 3′-deoxy-tRNA, tRNA in which the terminal adenosine has been replaced by 3′-deoxyadenosine; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

Preparation of α-[1-^{14}C]Aminobutyrate. The procedure used was a modification of the Strecker synthesis described by Loftfield & Eigner (1966). K^{14}CN (2.5 mCi) in 25 μL of water was added to propionaldehyde (20 μL) and 0.880 ammonia (25 μL) at −78 °C. The tube was sealed and allowed to stand at room temperature for 4 days. Concentrated HCl (300 μL) was added slowly with shaking and the liberated HCN removed by a gentle stream of nitrogen. After warming briefly at 100 °C, the tube was resealed and heated at 110 °C overnight. On completion of the hydrolysis, the contents of the tube were evaporated to dryness and chloroform (200 μL) was added to the dry residue. The α-D,L-aminobutyrate was extracted with water and then lyophilized to give about a 40% yield of crude material. Purification was accomplished by chromatography using SP Sephadex C-25. The column (1.5 × 10 cm) was equilibrated with 4 M acetic acid and the amino acid added in the same solution. After washing with 1 column volume of 4 M acetic acid, the amino acid was eluted with a gradient of 4 M acetic acid/0.04 M pyridine (100 mL, pH 2.5) to 1 M acetic acid/0.5 M pyridine (100 mL, pH 4.5). The racemate was resolved by preparing the chloroacetyl derivative and deacylating the L isomer with acylase I as described by Loftfield & Eigner (1966). The resultant mixture of α-L-aminobutyrate and *N*-chloroacetyl-α-D-aminobutyrate was separated using the above chromatographic procedure except that the column was washed with 40 mL of 4 M acetic acid and a shallower gradient of 300 mL, pH 2.5, pyridine–acetate/300 mL, pH 4.0, pyridine–acetate used. 500 μCi of α-[1-^{14}C]aminobutyrate (57.8 Ci/mol) was obtained. High voltage electrophoresis at pH 2.1 on Whatman no. 1 paper indicated a radiochemical purity of >99%. No radioactivity was found corresponding to [^{14}C]valine (<0.1%).

Preparation of [^{14}C]-αBut-tRNA^{Val} (E. coli). (a) A solution (300 μL) containing valyl-tRNA synthetase (*E. coli*, 50 μM), ATP (3.3 mM) α-[^{14}C]aminobutyrate (150 μM, 57.8 mCi/mmol) and inorganic pyrophosphatase (1 unit/mL) in the standard pH 7.78 buffer (144 mM Tris-Cl, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride) was incubated at 25 °C for 15 min. The solution was desalted on a column (1 × 10 cm) containing Sephadex G-25 (fine) equilibrated with the standard pH 5.87 buffer (13 mM Bistris-Cl replacing the Tris-Cl) at 4 °C to give 1.0 mL of solution containing 10 μM enzyme with 10 μM [^{14}C]-α-aminobutyryl adenylate bound. To this cold solution was added 10 nmol of tRNA^{Val} in 40 μL of 10 mM $MgCl_2$. The solution was immediately quenched with 1 mL of saturated phenol solution and the pH lowered to 5.0 with sodium acetate buffer (0.2 M). After centrifugation and separation, the tRNA was precipitated from the aqueous layer by the addition of 2 volumes of cold ethanol and standing at −20 °C. After desalting by gel filtration on Sephadex G-25 equilibrated with 10 mM $MgCl_2$, 9.5 nmol of tRNA^{Val} was obtained, 28% charged with [^{14}C]-αBut.

(b) A solution (340 μL) containing enzyme (71 μM), tRNA^{Val} (72 μM), ATP (8 mM), 1 unit of inorganic pyrophosphatase, and [^{14}C]-αBut (0.9 mM) in the standard pH 7.78 buffer was incubated for 2.5 min at 25 °C. After phenol extraction, ethanol precipitation and gel filtration as above, 95% of the tRNA was recovered, 5% of which was charged with [^{14}C]-αBut.

Preparation of [^{14}C]Val-tRNA^{Val} (E. coli or B. stearothermophilus). A solution (1.0 mL) containing tRNA^{Val} (46 μM), valyl-tRNA synthetase (5 μM), ATP (2 mM), [^{14}C]Val (55 μM, 270 Ci/mol), and 1 unit of inorganic pyrophosphatase in the standard pH 7.78 buffer was incubated for 2 min at room temperature. After the addition of 100 μL of 2 M pH 5.0 sodium acetate buffer, the solution was extracted with an equal volume of saturated phenol and the charged tRNA isolated by ethanol precipitation and gel filtration as above. tRNA^{Val} from *E. coli* was charged to 390 pmol/A_{260}, and gel filtration as above. tRNA^{Val} from *E. coli* was charged to 390 pmol/A_{260}, and from *B. stearothermophilus* to 1200 pmol/A_{260}.

Standard Conditions for Kinetic and Binding Experiments. All experiments were performed at 25 ± 0.1 °C in buffers containing 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and either 144 mM Tris-Cl at pH 7.78 or, where indicated, 13 mM Bistris-Cl at pH 5.87, unless otherwise stated.

Pyrophosphate exchange, aminoacylation and ATP pyrophosphatase activities were measured as described by Fersht & Kaethner (1976) and Fersht (1977a). The procedures for equilibrium gel filtration, nitrocellulose disk filter assays, and preparation of enzyme-bound aminoacyl adenylate complexes have been described previously (Mulvey & Fersht, 1977a). Solutions of trichloroacetic acid for quenching and precipitating tRNA contained the appropriate unlabeled amino acids.

Transient Formation of [^{14}C]-αBut-tRNA^{Val} (Bacterial). One syringe of the quenched flow apparatus (Fersht & Jakes, 1975) contained valyl-tRNA synthetase bound [^{14}C]-αBut-AMP (which had been freed from excess ligands by gel filtration) dissolved in the standard pH 5.87 buffer (13 mM Bistris-Cl). The other syringe contained tRNA in either the Bistris-Cl buffer or a more concentrated pH 7.78 buffer containing 288 mM Tris-Cl. The solutions were automatically mixed, incubated, and quenched with trichloroacetic acid. The precipitate was collected on nitrocellulose or glass fiber filters, dried, and assayed by scintillation counting. The concentration of enzyme-bound aminoacyl adenylate was periodically monitored by spotting 50 μL on presoaked nitrocellulose filters (Schleider and Schüll BA 85), washing with 3.0 μL of cold Bistris-Cl buffer, and, after drying, assaying by scintillation counting. The complex with the enzyme from *E. coli* was found to have a half-life of 82 min and that with *B. stearothermophilus* 400 min under the reaction conditions.

Transient Formation of [^{14}C]Thr-tRNA^{Val} (Yeast). The E·[^{14}C]Thr-AMP complex was formed in situ. One syringe of the quenched flow apparatus contained the enzyme from yeast in the standard pH 7.78 buffer with [^{14}C]Thr (232 Ci/mol), ATP (4 mM), and inorganic pyrophosphatase (1 unit/mL). The other syringe contained tRNA in the standard pH 7.78 buffer. The concentration of E·[^{14}C]Thr-AMP in the first syringe was periodically monitored by nitrocellulose filter assays. The [^{14}C]Thr-tRNA^{Val} which was transiently formed on mixing was assayed as above.

Valyl-tRNA Synthetase Catalyzed Deacylation of [^{14}C]-Val-tRNA^{Val}. To a solution (0.4 mL) of [^{14}C]Val-tRNA^{Val} (0.4 μM) in the buffered solution at 25 °C was added enzyme (35 μL of 50 μM). Aliquots (50 μL) were taken at 10–15-s intervals and quenched with 5% trichloroacetic acid, and the residual charged tRNA was assayed as above to establish the time course of deacylation. Experiments were repeated at different values of pH using buffers of ionic strength 0.1 (Tris-Cl, pH 7.4–7.78; Bistris-Cl, pH 6–7.0), and also in the presence of added inhibitors. The effect of preincubating the enzyme with valine, ATP, and inorganic pyrophosphatase to form the E·Val-AMP complex was noted. Both bacterial enzymes were used.

Valyl-tRNA Synthetase Catalyzed Deacylation of [^{14}C]-αBut-tRNA^{Val} (E. coli). One syringe of the quenched flow

Table I: Comparison of Pyrophosphate Exchange, Aminoacylation, and ATP Pyrophosphatase Activities of Bacterial Valyl-tRNA Synthetases[a]

reaction	amino acid	k_{cat} (s^{-1})	K_M (mM)
(a) *E. coli* enzyme			
pyrophosphate exchange[b]	Val	61	0.083
pyrophosphate exchange	αBut	25	6.5
aminoacylation[b]	Val	8.9	0.046
ATP pyrophosphatase	αBut	4.0[c]	0.6[c]
(b) *B. stearothermophilus* enzyme			
pyrophosphate exchange[d]	Val	33	0.03
pyrophosphate exchange	αBut	21	5.6

[a] pH 7.78, 25 °C, 10 mM $MgCl_2$, 2 mM ATP (+2 mM [^{32}P]-pyrophosphate for exchange reactions). [b] R. S. Mulvey, unpublished data. [c] [αBut] < 3.8 mM as inhibition occurs above this value. [d] Fersht & Kaethner, 1976.

apparatus contained valyl-tRNA synthetase (4 μM) in the appropriate buffer (at twice the normal buffer concentration); the other syringe contained $tRNA^{Val}$ (0.26 μM) and [^{14}C]-αBut-$tRNA^{Val}$ (0.10 μM) in unbuffered $MgCl_2$ solution (10 mM, pH ~5). The solutions were automatically mixed, quenched, and sampled to construct the time course for the deacylation. Experiments were repeated at varying values of pH, in the presence of α-aminobutyrate, and preincubating the enzyme with valine (or α-aminobutyrate), ATP, and inorganic pyrophosphatase to form the E·AA-AMP complex.

Results

Steady-State Kinetics. (a) Pyrophosphate Exchange. It is seen in Table I that the bacterial enzymes catalyze the pyrophosphate exchange reaction in the presence of α-aminobutyrate, in agreement with several earlier studies (Bergmann et al., 1961; Owens & Bell, 1970). In terms of the specificity constant k_{cat}/K_M, there is a selectivity of 200 times and 300 times respectively in favor of valine for the enzymes from the *E. coli* and *B. stearothermophilus*. This is similar to the selectivity of the isoleucyl-tRNA synthetase from *E. coli* for isoleucine in preference to valine.

(b) Aminoacylation vs. ATP/Pyrophosphatase Activity. The mischarging of tRNA with [^{14}C]-αBut was sought without success under a variety of conditions using *catalytic* quantities of enzyme and a large excess of tRNA. Instead, the two bacterial enzymes act as ATP/pyrophosphatases in the presence of α-aminobutyrate and tRNA. The value of k_{cat} for the ATP/pyrophosphatase reaction of the enzyme from *E. coli* is similar to that for aminoacylation when valine is present (Table I). A similar result was found previously when comparing the threonine dependent ATP/pyrophosphatase reaction of the enzyme from *B. stearothermophilus*.

Stoichiometry of Aminoacyl Adenylate Complex Formation. (a) Nonequilibrium Gel Filtration. Incubating the bacterial enzymes with a threefold excess of [^{14}C]-αBut (57.8 Ci/mol) in the presence of ATP and inorganic pyrophosphatase followed by gel filtration into the standard pH 5.87 buffer gave E·[^{14}C]-αBut-AMP complexes of stoichiometry about 0.9 in both cases. At pH 5.87 and 25 °C, the complex with the *E. coli* enzyme slowly hydrolyzed with a first-order rate constant of 1.4×10^{-4} s^{-1}, and that with the enzyme from *B. stearothermophilus* with a rate constant of 3×10^{-5} s^{-1}. The complex of the *E. coli* enzyme with [^{14}C]Thr-AMP was formed with lower stoichiometry (Mulvey & Fersht, 1977a).

(b) Equilibrium Gel Filtration. Adding 100 μL of a 3 μM solution of the valyl-tRNA synthetase from *B. stearothermophilus* to a 1-mL column equilibrated with 15 μM [^{14}C]-αBut, 2 mM ATP, and 1 unit/mL of inorganic pyrophosphatase (Mulvey & Fersht, 1977a) and monitoring the passage of enzyme and radioactivity showed that a complex of stoichiometry 0.94 was formed.

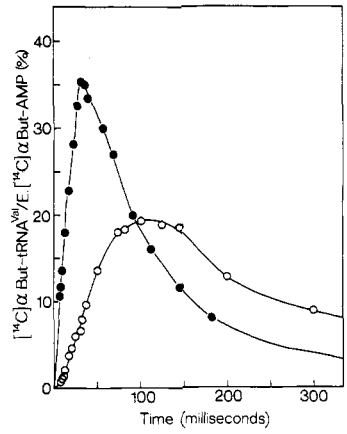

FIGURE 1: Transient formation of [^{14}C]-αBut-$tRNA^{Val}$ during the rejection of α-aminobutyrate by the valyl-tRNA synthetase from *B. stearothermophilus* at 25 °C detected by mixing equal volumes of E·[^{14}C]-αBut-AMP and $tRNA^{Val}$ in the quenched flow apparatus. (○) pH 5.87, 0.9 μM E·[^{14}C]-αBut-AMP, 8.6 μM $tRNA^{Val}$; (●) pH 7.78, 0.9 μM E·[^{14}C]-αBut-AMP, 8.6 μM $tRNA^{Val}$ (concentrations after mixing).

(c) Nitrocellulose Disk Filter Assays. An E·[^{14}C]-αBut-AMP complex of stoichiometry 0.95 was formed with the enzyme from *B. stearothermophilus* compared with a value of 0.99 for a control using [^{14}C]Val. The corresponding values for the *E. coli* enzyme were 0.94 and 0.93.

Trapping of [^{14}C]-αBut-$tRNA^{Val}$ *Using Substrate Quantities of Enzyme.* (a) Preparatively from E·[^{14}C]-αBut-AMP. On mixing equal quantities of aminoacyl adenylate complex and $tRNA^{Val}$ from *E. coli* at 0 °C and pH 5.9 and quenching as rapidly as possible with phenol, 28% of the $tRNA^{Val}$ was found to be charged with [^{14}C]-αBut. Much smaller yields were obtained from the *B. stearothermophilus* system.

(b) Preparatively from Enzyme, tRNA, [^{14}C]-αBut, and ATP in the Steady State. After incubating a mixture of [^{14}C]-αBut (0.9 mM), ATP, inorganic pyrophosphatase, and equal quantities of valyl-tRNA synthetase and $tRNA^{Val}$ from *E. coli* for 2.5 min in the standard pH 7.78 buffer and then quenching and extracting the tRNA with phenol, 5% of the tRNA was found to be aminoacylated with [^{14}C]-αBut. The deacylation of this material was found to be catalyzed by the valyl-tRNA synthetase with a turnover number of 50 s^{-1}, the same as for the material isolated in a above.

A steady-state concentration of [^{14}C]-αBut-$tRNA^{Val}$ (*B. stearothermophilus*) was similarly detected by trichloroacetic acid precipitation from a reaction mixture containing 5 μM enzyme, 5 μM tRNA, 400 μM [^{14}C]-αBut, 10 mM ATP, and 5 units/mL of inorganic pyrophosphatase incubated in the standard pH 7.78 buffer at 25 °C. The level of [^{14}C]-αBut-$tRNA^{Val}$ remained at 4.8% of the $tRNA^{Val}$ (or enzyme) from 15 s to 10 min. Doubling the concentration of [^{14}C]-αBut increased the level of [^{14}C]-αBut-$tRNA^{Val}$ to 5.6%. Under these conditions using 460 μM αBut and 10 mM [γ^{32}P]ATP, the measured ATP/pyrophosphatase activity (v/[E]) is 0.7 s^{-1}.

(c) Transient Formation from E·[^{14}C]αBut-AMP at 25 °C Using the Quenched-Flow Apparatus. It is seen in Figure 1 that on mixing E·[^{14}C]-αBut-AMP with $tRNA^{Val}$ (*B. stearothermophilus*) there is a large, transient, formation of [^{14}C]-αBut-$tRNA^{Val}$. At pH 7.78, 35% of the radioactivity is present as [^{14}C]-αBut-$tRNA^{Val}$ at the maximum (32–36

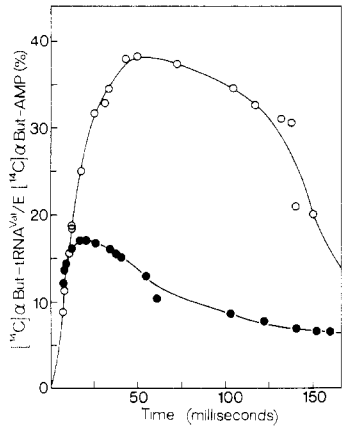

FIGURE 2: Transient formation of [^{14}C]-αBut-tRNA^{Val} during the rejection of α-aminobutyrate by the valyl-tRNA synthetase from *E. coli* at 25 °C (as in Figure 1). (○) pH 5.87, 0.9 μM E·[^{14}C]-αBut-AMP, 11 μM tRNA^{Val}; (●) pH 7.78, 1.2 μM E·[^{14}C]-αBut-AMP, 1.7 μM tRNA^{Val}.

ms). At pH 5.87, there is a lower maximum of about 19% at 100 ms. The early time course at pH 5.87 suggests there is a "lag" in the transfer reaction. This might be caused by a rate-determining conformational change on tRNA binding. Large transfers of [^{14}C]-αBut to tRNA^{Val} are also observed in Figure 2, for the reactions of the enzyme and tRNA from *E. coli*. At pH 7.78, the maximum is 17% transfer at 17–21 ms; at pH 5.87, the maximum is 38% transfer at 50 ms. Because of the complex nature of the reactions, the traces being at least triphasic, no attempt was made to fit the reactions to an analytical solution. But simple extrapolation of the data at pH 5.87 for *E. coli* and at pH 7.78 for *B. stearothermophilus*, allowing for the rapid hydrolysis of the mischarged tRNA, indicates that most of the [^{14}C]-αBut from the E·[^{14}C]-αBut-AMP complex is transiently transferred to tRNA^{Val}. The lower maxima found at the other values of pH presumably reflect a lower rate of formation of the mischarged tRNA since the rate of hydrolysis is independent of pH.

Trapping of [^{14}C]-Thr-tRNA^{Val} (Yeast) Using Substrate Quantities of Enzyme. Insufficient quantities of the valyl-tRNA synthetase from yeast were available for preliminary studies on the isolation and stability of the E·[^{14}C]Thr-AMP complex. The complex was therefore produced in situ by incubating the enzyme in a buffer containing [^{14}C]Thr, ATP, and inorganic pyrophosphatase (1 unit/mL) and monitoring the concentration by nitrocellulose disk filtration. On mixing the complex with tRNA in the quenched-flow apparatus, there is a substantial transient formation of [^{14}C]Thr-tRNA^{Val}. It is seen in Figure 3 that the amount at the maximum increases significantly with increasing concentration of tRNA. The rate of the transfer reaction is sufficiently high that it is probably limited by the rate of association of the tRNA with the enzyme at these concentrations. Some of the increase in the amount of Thr-tRNA^{Val} must be caused by an increase in formation rate with increasing concentration of tRNA. Complications must also arise from the subspecies of tRNA^{Val} in the unfractionated sample.

This is the first direct evidence that the yeast valyl-tRNA synthetase rejects threonine by transient misacylation of the tRNA. Further, it may be estimated from the decay regions of the curves in Figure 3 that the rate constant for the deacylation of E·Thr-tRNA^{Val} complex is at least 80 s^{-1}, some two and three orders of magnitude above the steady-state values measured by Igloi et al. (1977). During the transfer reaction, the enzyme is a far more potent esterase than indicated from steady-state kinetics using synthetically mischarged tRNA.

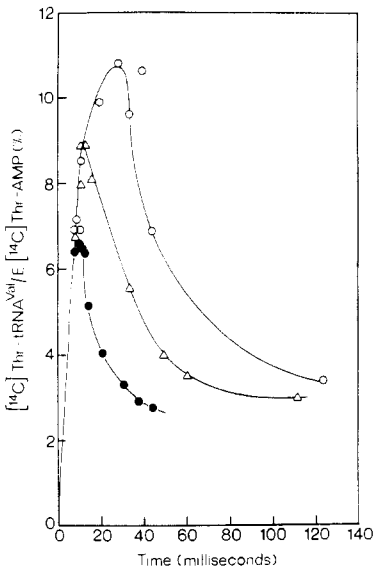

FIGURE 3: Transient formation of [^{14}C]Thr-tRNA^{Val} during the rejection of threonine by the valyl-tRNA synthetase from yeast at 25 °C and pH 7.78. Equal volumes of tRNA solution and E·[^{14}C]Thr-AMP (formed in situ) were automatically mixed and sampled in the quenched flow apparatus. (○) 9.4 μM tRNA^{Val}, 0.12 μM enzyme, 7.2 μM [^{14}C]Thr, and 2 mM ATP; (△) 4.7 μM tRNA^{Val}, 0.15 μM enzyme, 0.54 μM [^{14}C]Thr, and 2 mM ATP; (●) 4.7 μM tRNA^{Val}, 0.24 μM enzyme, 0.54 μM [^{14}C]Thr, and 2 mM ATP (concentration after mixing; solutions also contained inorganic pyrophosphatase).

Deacylation Reactions. (a) Val-tRNA^{Val}. The enzyme-catalyzed deacylation reaction was performed under single turnover conditions of [E] ≫ [S] and [E] > K_M. The rate constant measured is for the process:

$$\text{E·Val-tRNA}^{Val} \rightarrow \text{E·Val·tRNA}^{Val} \quad (2)$$

Under these conditions, the kinetics are not complicated by the rate constants for the dissociation of the E·tRNA complexes or by any competitive inhibition by uncharged tRNA. Excellent first-order plots were obtained (Figure 4, lower panel). The rate constants varied with pH according to the ionization of a base of pK_a = 8 for both the *E. coli* and *B. stearothermophilus* enzymes, the latter rate constants being generally 2.2 times faster (data not shown). The rate constant for the *B. stearothermophilus* enzyme is 4.3 × 10^{-2} s^{-1} at pH 7.78 and 8.5 × 10^{-4} s^{-1} at pH 6.0. The limiting velocity at high pH is about 0.11 s^{-1}. The rate constants for the *E. coli* system are 2 × 10^{-2} s^{-1} at pH 7.78 and 3.8 × 10^{-4} s^{-1} at pH 6.0, with a limiting velocity of 5 × 10^{-2} s^{-1} at high pH. Preincubation of the enzyme in buffered valine, ATP, and inorganic pyrophosphatase to form the E·Val-AMP complex lowered the rate constant 5-fold at pH 7.78 for *B. stearothermophilus*, and 2.4-fold for *E. coli*. This type of behavior is similar to that reported by Eldred & Schimmel (1972) for the deacylation of Ile-tRNA^{Ile}.

(b) αBut-RNA^{Val}. The pH dependence and inhibition of the error correcting deacylation for the *E. coli* system are quite different from the above. It is seen in Figure 4 (upper panel) that, under the single turnover conditions of [E] ≫ [αBut-tRNA], first-order kinetics are obeyed with a single rate constant of 50 s^{-1} from pH 6 to 7.8 and that this is unaffected by the conversion of E to E·Val-AMP.

The rate-determining step of the editing mechanism is clearly different from that of the hydrolysis of the cognately charged tRNA. The hydrolysis of Val-tRNA^{Val} is either an entirely different enzymic process from that of editing or, alternatively, the nonspecific deacylation of Val-tRNA^{Val} is

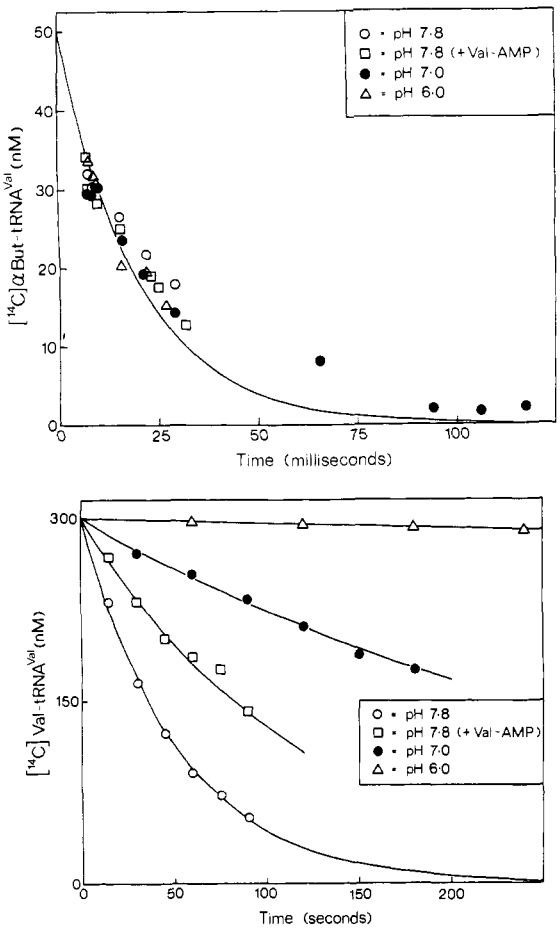

FIGURE 4: Deacylation reactions catalyzed by the valyl-tRNA synthetase from *E. coli* at 25 °C. Lower: hydrolysis of [^{14}C]Val-tRNA^{Val} catalyzed by 4 μM enzyme. Upper: hydrolysis of [^{14}C]-αBut-tRNA^{Val} catalyzed by 2 μM enzyme. Solid curves are theoretical first-order plots.

an attenuated version of the editing deacylation in which a different step has become rate determining.

The incorrectly charged αBut-tRNA^{Val} is deacylated some 2.5×10^3 times more rapidly at pH 7.78 and 1.3×10^5 times at pH 6.0 than is the cognately charged Val-tRNA^{Val}.

Product inhibition by αBut of the deacylation of αBut-tRNA could not be found at up to 38 mM αBut. The E·αBut-AMP complex was found to be as hydrolytically active as the free enzyme.

Discussion

The rejection of α-aminobutyrate by the valyl-tRNA synthetases from *B. stearothermophilus* and *E. coli* is very similar to the rejection of threonine which we have described previously (Fersht & Kaethner, 1976). The enzymes activate the noncognate amino acid but discriminate against it by binding it more weakly than valine. Relatively stable E·αBut-AMP complexes of unit stoichiometry are formed in which the αBut-AMP is sequestered against hydrolysis. Error correction occurs on the addition of tRNA^{Val} to the compexes; they are hydrolytically destroyed. During the hydrolytic reaction, αBut-tRNA^{Val} is formed. This was detected by rapid quenching experiments and isolated on a preparative scale by phenol quenching and extraction at pH 6 and 0 °C. A 28% yield of [^{14}C]-αBut-tRNA^{Val} was obtained on the addition of equimolar quantities of tRNA^{Val} and the E·[^{14}C]-αBut-AMP complex from *E. coli*. The mischarged tRNA is deacylated with a value of 50 s^{-1} for k_{cat}. The net result of the misacylation/deacylation pathway is that, in the presence of tRNA^{Val} and α-aminobutyrate, the valyl-tRNA synthetases act as ATP/pyrophosphatases. The enzyme from *E. coli* has a value of k_{cat} of 4 s^{-1} and a K_M of 0.6 mM for α-aminobutyrate in this reaction. The kinetic interpretation is that αBut-tRNA^{Val} is formed with these values for k_{cat} and K_M, and is then rapidly deacylated with a rate constant of 50 s^{-1}. These data may be used to calculate steady-state concentrations of αBut-tRNA^{Val}.

αBut-tRNA^{Val} Is a Kinetically Competent Intermediate in the Steady State. The nature of the editing mechanism for the rejection of threonine by the valyl-tRNA synthetase from *B. stearothermophilus* was established by the following procedure (Fersht & Kaethner, 1976). (1) Thr-tRNA^{Val} was shown to be a kinetically competent intermediate in the destruction of E·Thr-AMP on the addition of tRNA^{Val}: the Thr-tRNA^{Val} was isolated during the reaction, characterized, shown to react fast enough and to be formed fast enough for the overall reaction. (2) The E·Thr-AMP·tRNA^{Val} complex was shown to be formed in the steady-state by pyrophosphate exchange kinetics. The same has been shown here for the rejection of α-aminobutyrate. Further, we have now provided *direct* evidence, by isolation of the intermediate, that αBut-tRNA^{Val} is formed during the steady-state reaction. Using the above values of k_{cat} and K_M for the ATP/pyrophosphatase reaction (to estimate the rate of misacylation) and the value of 50 s^{-1} for the deacylation step, it is calculated that, at 0.9 mM [^{14}C]-αBut (plus ATP, tRNA etc.), 4.8% of the enzyme is in the form E·[^{14}C]-αBut-tRNA (eq 3). This amount of

$$\text{E·ATP·tRNA}^{Val} \xrightarrow[2.4\ s^{-1}]{\alpha\text{But}} \text{E·}\alpha\text{But-tRNA}^{Val} \xrightarrow{50\ s^{-1}} \text{E} + \alpha\text{But} + \text{tRNA}^{Val} \quad (3)$$

misacylated tRNA was isolated from a reaction mixture containing 71 μM enzyme and 72 μM tRNA^{Val} (*E. coli*).

This simple, direct, experiment was difficult to perform previously for the case of rejection of threonine since the high K_M for threonine in the ATP/pyrophosphatase reaction (7 mM, Fersht & Kaethner, 1976) required very high concentrations of [^{14}C]Thr for the observable accumulation of Thr-tRNA^{Val}. Further complications arose from the presence of trace impurities of threonyl-tRNA synthetase and tRNA^{Thr} in the enzyme and tRNA preparations, and impurities in the commercially obtained amino acid. There are no such difficulties using [^{14}C]αBut: it was synthesized by us in large amounts and in high purity; there is no α-aminobutyryl-tRNA synthetase and tRNA$^{\alpha But}$ to complicate the issue.

Generalization of the Misacylation/Deacylation Mechanism for Valyl-tRNA Synthetases. On addition of tRNA to the valyl-tRNA synthetase (yeast)·threonyl adenylate complex, Thr-tRNA^{Val} is transiently formed and is deacylated with a rate constant of 80 s^{-1} or greater (Figure 3). The direct detection of the intermediate and the finding of the rapid hydrolysis rate remove doubts about the misacylation/deacylation pathway raised by the reported value of 0.5 s^{-1} for k_{cat} for the enzyme-catalyzed deacylation of (synthetic) Thr-tRNA^{Val} in the steady state (Igloi et al., 1977). Why the steady-state value is so much lower is not known. Perhaps the enzyme is in a more active conformation immediately following aminoacyl-tRNA formation during the aminoacylation reaction, or, in the steady-state hydrolytic reaction, there is the slow release of products that becomes rate limiting. Whatever the explanation, both prokaryotic and eukaryotic valyl-tRNA synthetases edit errors of misrecognition of amino acids by rapidly deacylating the mischarged tRNA.

Deacylation Reactions. The editing hydrolytic reaction, the deacylation of αBut-tRNA^{Val} catalyzed by the valyl-tRNA

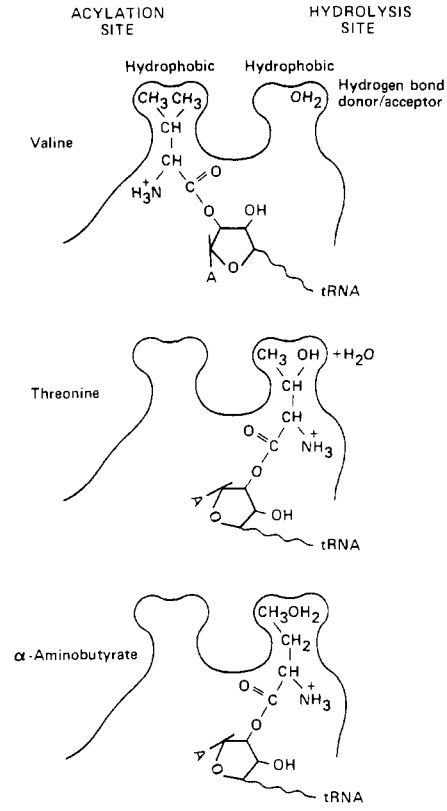

FIGURE 5: Scheme for specificity in deacylation (see text). The aminoacyl moiety translocates to the hydrolytic site either by the movement of the terminal adenosine as shown, or possibly by a 2′→3′-acyl transfer.

synthetase from *E. coli*, differs from "nonspecific hydrolysis", the deacylation of Val-tRNA^{Val}, in two respects (Figure 4): (a) the rate constant for the editing reaction is pH independent (between pH 6 and pH 7.8) and (b) it is unaffected by the addition of Val-AMP to the enzyme; the rate constant for the hydrolysis of Val-tRNA^{Val} increases with pH according to the ionization of a base of $pK_a = 8$, and is decreased two- to fivefold on addition of Val-AMP. At pH 7, the editing reaction is 10^4 times faster than the nonspecific reaction.

The mechanism of the deacylation in the editing reaction is unknown. Igloi et al. (1977) suggest that, for the deacylation of αBut-tRNA^{Val}, a water molecule is bound in the position normally occupied by the additional methylene group of Val-tRNA^{Val}, and that this water molecule is activated to attack the ester bond, as proposed earlier for the deacylation of Val-tRNA^{Ile} catalyzed by the isoleucyl-tRNA synthetase (von der Haar & Cramer, 1976). An additional mechanism is required for the deacylation of Thr-tRNA^{Val} and it is suggested that the –OH group of threonine is involved chemically (Igloi et al., 1977). These proposals appear to be ruled out, however, by the finding that blocking the aminoacylation site of the valyl-tRNA synthetase with valyl adenylate does not affect the rate of the editing reaction. This implies that the aminoacyl moiety of the mischarged tRNA translocates from the aminoacylation site to a separate hydrolytic site. (Against this proposal, it may, perhaps, be argued that the valyl adenylate in the isolated complex binds to a site distant from the normal aminoacylation site, and leaves it open for the deacylation reaction. We consider this possibility unlikely, since, in all cases we have studied so far, the addition of tRNA to an isolated enzyme·aminoacyl adenylate complex leads to the formation of aminoacyl-tRNA at a rate comparable with that of the reaction in the steady state. Such a high rate, we feel, argues against any translocation of Val-AMP to the aminoacylation site.)

A unified mechanism for selection in the editing mechanism is presented in Figure 5, an extension of that proposed previously (Fersht & Kaethner, 1976). It is possible that the hydrolytic site contains a hydrogen bond donor/acceptor which is normally bound to a water molecule. It is energetically unfavorable for valine to occupy this site since the hydrogen-bonded water molecule would be displaced by the hydrophobic methyl group. However, the –OH group of threonine could replace the water molecule on binding. Further, α-aminobutyrate (or any other smaller amino acid) could bind without displacing the water molecule. Selectivity in deacylation is thus achieved by discriminating against a methylene group of valine by means of its hydrophobic nature.

The question may be asked why the enzyme edits the mistaken incorporation of the unnatural amino acid α-aminobutyrate. This amino acid is in fact found in vivo (e.g., Morgan et al., 1977). But we consider that this editing is also a manifestation of a general editing function of the valyl-tRNA synthetase which removes the mistaken activation of amino acids smaller than valine.

Significance of the Slow Deacylation Rates Found by Bonnet & Ebel (1974). The major problem in specificity is the rejection of substrates that are smaller than or isosteric with the correct substrate, i.e., those substrates which cannot be excluded by steric hindrance (Fersht, 1977b). The editing mechanism is required to remove the products of only these substrates—the "double-sieve" analogy (Fersht, 1977b, p 283). All the examples of misacylated tRNA studied by Bonnet & Ebel (1974) were aminoacylated with substrates *larger* than the correct amino acid (or having a branched side chain instead of an unbranched side chain). It is precisely this class of misacylation that is expected to be resistant to hydrolytic editing.

References

Baldwin, A. N., & Berg, P. (1966) *J. Biol. Chem. 241*, 839.
Bergmann, F. H., Berg, P., & Dieckmann, H. (1961) *J. Biol. Chem. 236*, 1735.
Bonnet, J. (1974) *Biochimie 56*, 541.
Bonnet, J., & Ebel, J. P. (1974) *FEBS Lett. 39*, 259.
Eldred, E. W., & Schimmel, P. R. (1972) *J. Biol. Chem. 247*, 2961.
Fersht, A. R. (1977a) *Biochemistry 16*, 1025.
Fersht, A. R. (1977b) *Enzyme Structure and Mechanism*, Chapter 11, W. H. Freeman & Co., San Francisco.
Fersht, A. R., & Jakes, R. (1975) *Biochemistry 14*, 3350.
Fersht, A. R., & Kaethner, M. M. (1976) *Biochemistry 15*, 3342.
Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L. E., & Hartley, B. S. (1975) *Biochemistry 14*, 1.
Igloi, G. L., von der Haar, F., & Cramer, F. (1977) *Biochemistry 16*, 1696.
Loftfield, R. B., & Eigner, E. A., (1966) *Biochim. Biophys. Acta 130*, 449.
Loftfield, R. B., & Vanderjagt, D. (1972) *Biochem. J. 128*, 1353.
Morgan, M. Y., Milson, J. P., & Sherlock, S. (1977) *Science 197*, 1183.
Mulvey, R. S., & Fersht, A. R. (1977a) *Biochemistry 16*, 4005.
Mulvey, R. S., & Fersht, A. R. (1977b) *Biochemistry 16*, 4731.
Norris, A., & Berg, P. (1965) *Proc. Natl. Acad. Sci. U.S.A. 52*, 330.
Owens, S. L., & Bell, F. E. (1970) *J. Biol. Chem. 245*, 5515.

Sourgoutchoff, A., Blanquet, S., Fayat, G., & Waller, J. P. (1974) *Eur. J. Biochem. 46*, 431.

von der Haar, F., & Cramer, F. (1976) *Biochemistry 16*, 4131.

Yarus, M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1915.

Cysteinyl-tRNA Synthetase from *Escherichia coli* Does Not Need an Editing Mechanism to Reject Serine and Alanine. High Binding Energy of Small Groups in Specific Molecular Interactions†

Alan R. Fersht* and Colin Dingwall

ABSTRACT: The cysteinyl-tRNA synthetase from *Escherichia coli* only very slowly activates serine, alanine, and α-aminobutyrate, the possible competitors of cysteine. The upper limits on the values of k_{cat}/K_M for the amino acid dependent ATP/pyrophosphate exchange reactions, relative to that of cysteine, are $<10^{-8}$, 2×10^{-7}, and 3×10^{-6}, respectively. It is calculated from these data and the concentrations of the amino acids in vivo that the error rates for the misincorporation of serine and alanine for cysteine are less than 10^{-9} and 5×10^{-8}, respectively. There is no need for an error correcting mechanism and no evidence has been found to implicate one: there is no detectable ATP/pyrophosphatase activity of the enzyme in the presence of $tRNA^{Cys}$ and alanine; Ala-$tRNA^{Cys}$ has been synthesized by the reductive desulfurization of Cys-$tRNA^{Cys}$ and has been found to be relatively resistant to the enzyme-catalyzed deacylation. Part of the high selectivity of the enzyme for the –SH group of cysteine (~5 kcal/mol) appears to be caused by dispersion forces: simple calculations suggest that the dispersion energy between sulfur and a methylene group is about 2.5 times greater than that between two methylene groups. This high "hydrophobicity" of sulfur is consistent with the relative binding energies of substrates of the methionyl-tRNA synthetase. The rest of the high binding energy of the –SH group may come from hydrogen bonding.

Not all aminoacyl-tRNA synthetases are faced with severe problems in rejecting competing amino acids. Certain amino acids are sufficiently different in structure from their nearest competitors that adequate specificity may be provided by simple binding. Phenylalanine, for example, binds at least 3×10^4 times more weakly than tyrosine to the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* (Fersht et al., 1975) because phenylalanine lacks the hydrogen bonding potential of the phenolic hydroxyl. In this context, it is noted that the tyrosyl-tRNA synthetase is one of the few aminoacyl-tRNA synthetases that will aminoacylate either the 2′- or 3′-terminal hydroxyl groups of the cognate tRNA (Cramer et al., 1975). The valyl- and isoleucyl-tRNA synthetases are specific for the 2′ hydroxyl in aminoacylation (Cramer et al., 1975; Sprinzl & Cramer, 1975; Chinault et al., 1976) but require the nonaccepting 3′ hydroxyl in some way for the hydrolytic editing (von der Haar & Cramer, 1975; Igloi et al., 1977). It has been suggested that the aminoacylation and editing sites are on either side of the two terminal hydroxyl groups (Fersht & Kaethner, 1976). The lack of specificity for the 2′- or 3′-hydroxyl group would be consistent with the notion that the tyrosyl-tRNA synthetase also lacks a hydrolytic site for the deacylation of mischarged tRNA.

The cysteinyl-tRNA synthetase is another example that will aminoacylate both the 2′- and 3′-hydroxyl groups. There is the experimental advantage with this enzyme that the sulfur of its correctly charged cognate tRNA may be removed reductively with Raney nickel to produce the mischarged species, Ala-$tRNA^{Cys}$ (Chapeville et al., 1962). Alanine, being smaller than cysteine by one sulfur atom, is one of the most likely competitors of cysteine for the active site of the cysteinyl-tRNA synthetase.

In this paper, we have determined the specificity of the cysteinyl-tRNA synthesis in the pyrophosphate exchange reaction for cysteine, alanine, serine and α-aminobutyrate to determine whether adequate selection is possible without a subsequent deacylating mechanism. We have prepared Ala-$tRNA^{Cys}$ to see whether it is rapidly deacylated by the enzyme in a possible error correcting step.

Experimental Section

Materials. The cysteinyl-tRNA synthetase was isolated from *Escherichia coli* as described elsewhere (Mulvey et al., 1978). Partially purified $tRNA^{Cys}$ (amino acid acceptance = 30 pmol/A_{260} unit) was obtained from the Microbiological Research Establishment, Porton Down, Wiltshire, England. Radioactively labeled amino acids were obtained from the Radiochemical Centre, Amersham, England. Unlabeled amino acids were obtained from Sigma or BDH and further purified by recrystallization from ethanol–water.

The concentration of the cysteinyl-tRNA synthetase was calculated using a mol wt of 59000 and a value of 2 cm^{-1} mg^{-1} mL^{-1} for A_{280} (Mulvey et al., 1978). Activity was routinely checked by active site titration (Fersht et al., 1975), and purity was evaluated by polyacrylamide gel electrophoresis using sodium dodecyl sulfate.

Preparation of [^3H]*Cys-tRNA*^{Cys}. A solution (0.68 mL) of [3,3′-^{3}H]cystine (74 μM, 1.6 Ci/mmol), Tris-Cl (pH 7.8, 0.64 M), and dithiothreitol (44 mM) was incubated at 25 °C to reduce the cystine to cysteine. After 15 min, tRNA (250 A_{260} units/mL), $MgCl_2$ (10 mM), ATP (2 mM), phenylmethanesulfonyl fluoride (0.1 mM), inorganic pyrophosphatase (0.3 units/mL), and cysteinyl-tRNA synthetase (0.5 μM) were added to give a total volume of 3 mL. After a further 10 min, the pH was lowered to 5 by the addition of sodium acetate buffer (0.2 M) and the solution extracted with an equal volume

† From the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, England. *Received June 13, 1978; revised manuscript received December 11, 1978.*

* Address from Sept 1978 to Sept 1979: Department of Biochemistry, Stanford University Medical Center, Stanford, CA 94305.

0006-2960/79/0418-1245$01.00/0 © 1979 American Chemical Society