in tubulin as the protein undergoes polymerization. The reason why denaturation of microtubules did not make all the thiols available to the mercurial reagent is not known. The disulfide bridge of platelet tubulin was accessible to DTNB only in the uncoiled form of the protein. Our results show that polymerization did not result in formation of interchain disulfide bridges as polymerized tubulin had the same number of disulfide bonds as depolymerized tubulin.

Sulfhydryl groups are clearly involved in the assembly of microtubules. In platelet tubulin 3 sulfhydryl groups appear to be involved in the formation of microtubules. Kuriyama & Sakai (1974) reported a similar behavior for tubulin of porcine brain in which the blocking of 2 sulfhydryl groups abolished its polymerizability. The complete reversal by DTT of the inhibitory effect of PCMB on the ability to form microtubules confirms the importance of free sulfhydryls for this process. In sharp contrast is the effect of sulfhydryl blocking agents on the colchicine binding activity of tubulin. This phenomenon which occurs only when tubulin has a specific conformation (Ventilla et al., 1972) seems to be little affected by free sulfhydryl groups. As the heterodimer is essential for ligand activity, one can conclude that sulfhydryls are not critical for the interaction of the 2 tubulin subunits.

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# Aminoacyl-tRNA Synthetases from Yeast: Generality of Chemical Proofreading in the Prevention of Misaminoacylation of tRNA†

Gabor L. Igloi,* Friedrich von der Haar, and Friedrich Cramer

ABSTRACT: The specificity of valyl-, phenylalanyl-, and tyrosyl-tRNA synthetases from yeast has been examined by a series of stringent tests designed to eliminate the possibility of artefactual interference. Valyl-tRNA synthetase, as well as activating a number of amino acid analogues, will accept alanine, cysteine, isoleucine, and serine in addition to threonine as substrates for both ATP-$PP_i$ exchange and transfer to some $tRNA^{Val}$ species. The transfer is not observed if attempts are made to isolate the appropriate aminoacyl-$tRNA^{Val}$-C-C-A but its role in the overall aminoacylation can be suspected from both the formation of a stable aminoacyl-$tRNA^{Val}$-C-C-A(3′$NH_2$) compound and from the stoichiometry of ATP hydrolysis during the aminoacylation of the native tRNA. Similar tests with phenylalanyl-tRNA synthetase indicate that this enzyme will also activate and transfer other naturally occurring amino acids, namely, leucine, methionine, and tyrosine. The tyrosine enzyme, which lacks the hydrolytic capacity of the other two enzymes (von der Haar, F., & Cramer, F. (1976) *Biochemistry 15,* 4131-4138) is probably absolutely specific for tyrosine. It is concluded that chemical proofreading, in terms of an enzymatic hydrolysis of a misacylated tRNA, plays an important part in maintaining the specificity in the overall reaction and that this activity may be more widespread than has so far been suspected.

The importance of fidelity in the aminoacylation of tRNAs has been appreciated for a long time (Pauling, 1958) but proposals have only recently been put forward for the way in which specificity is maintained (for a review, see Igloi & Cramer, 1978). Such mechanisms, in whichever detailed form one expresses them, are a necessary property of protein synthesizing systems if only to reduce the well-known isoleucyl-tRNA synthetase/valine misactivation (Baldwin & Berg, 1966) which, although efficient in the isolated systems, results in maximally 1 error in 3000 in the presence of the complex protein synthesizing machinery (Loftfield & Vanderjagt, 1972). Has the intricate mechanism of chemical proofreading (von der Haar & Cramer, 1976) which brings about the reduction in the error rate in the isoleucyl-tRNA synthetase/

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TABLE I: Amino Acid Analogues Activated by Various Aminoacyl-tRNA Synthetases.

| analogues | ref[a] | analogues | ref[a] |
|---|---|---|---|
| valyl-tRNA synthetase | | prolyl-tRNA synthetase | |
| allo-α-amino-β-chlorobutyric acid | 5 | allo-4-hydroxyproline | 24 |
| L-alloisoleucine | 1 | L-azetidine-2-carboxylic acid | 8, 23–25, 27 |
| L-α-aminobutyric acid | 1–6, 8 | 3,4-dehydro-DL-proline | 23 |
| α-amino-β-chlorobutyric acid | 3, 5 | *N*-ethylglycine | 24 |
| cyclobutylglycine | 7, 32 | *N*-methylalanine | 24 |
| cycloleucine | 6, 8, 31 | *N*-methylglycine | 24 |
| cyclopropylglycine | 7, 18, 31 | *trans*-3-methylproline | 24 |
| DL-homoserine | 31 | *N*-propylglycine | 24 |
| L-*O*-methylthreonine | 4 | thiazolidene-4-carboxylic acid | 23, 24 |
| L-norvaline | 6, 31 | | |
| L-penicillamine | 31, 33 | lysyl-tRNA synthetase | |
| | | *trans*-4-dehydrolysine | 18 |
| phenylalanyl-tRNA synthetase | | 2,6-diamino-4-hexynoic acid | 18 |
| *trans*-2-amino-4-heptenoic acid | 27, 29 | 4-oxalysine | 18 |
| L-2-aminohexa-4,5-dienoic acid | 28 | thiosine | 17 |
| DL-2-amino-4-methylhex-4-enoic acid | 28 | | |
| L-2-amino-5-methylhex-4-enoic acid | 28 | tyrosyl-tRNA synthetase | |
| DL-crotylglycine | 27, 28 | 3-fluorotyrosine | 21 |
| cyclopentenealanine | 27 | 3-hydroxytyrosine | 21 |
| cyclohexenealanine | 27 | 5-hydroxy-2-(3-DL-alanyl)pyridine | 21 |
| 3,4-difluorophenylalanine | 27 | D-tyrosine | 21 |
| DL-ethallylglycine | 28 | | |
| 2-fluorophenylalanine | 29 | leucyl-tRNA synthetase | |
| DL-3-fluorophenylalanine | 28, 29 | azaleucine | 7 |
| 4-fluorophenylalanine | 27, 29 | cyclopropanealanine | 18 |
| DL-methallylglycine | 28 | cyclopropaneglycine | 18 |
| DL-4-methoxyphenylalanine | 28 | L-γ-hydroxynorvaline | 7 |
| mimosine | 30 | L-hypoglycine | 7 |
| DL-β-phenylserine | 27, 28 | methallylglycine | 18 |
| DL-β-pyrazol-1-ylalanine | 28 | norleucine | 7 |
| DL-β-pyrid-2-ylalanine | 27, 28 | | |
| 4-thiazolealanine | 27 | methionyl-tRNA synthetase | |
| DL-β-thien-2-ylalanine | 27, 28 | α-aminobutyric acid | 16 |
| | | ethionine | 15, 16 |
| isoleucyl-tRNA synthetase | | DL-homocysteine | 16 |
| L-alloisoleucine | 1 | norleucine | 15, 16 |
| DL-α-aminoheptanoic acid | 14 | norvaline | 16 |
| L-*O*-methylthreonine | 13, 14 | | |
| DL-norleucine | 14 | tryptophanyl-tRNA synthetase | |
| L-norvaline | 1, 14 | 7-azatryptophan | 20 |
| | | 4-fluorotryptophan | 19 |
| arginyl-tRNA synthetase | | 5-fluorotryptophan | 19, 20 |
| canavanine | 22 | 6-fluorotryptophan | 19 |
| homoarginine | 22 | 7-fluorotryptophan | 19 |

[a] References are as follows: (1) Loftfield & Eigner (1966); (2) George & Meister (1967); (3) Freundlich (1967); (4) Igloi et al. (1977); (5) Bergmann et al. (1961); (6) Owens & Bell (1970); (7) Anderson & Fowden (1970b); (8) Chuang et al. (1967); (9) Yaniv & Gros (1969); (10) Kondo & Woese (1969); (11) Berg et al. (1961); (12) Arca et al. (1967); (13) Smulson & Rabinovitz (1968); (14) Holler et al. (1973); (15) Trupin et al. (1966); (16) Old & Jones (1977); (17) Stern & Mehler (1965); (18) Harding & DeShazo (1967); (19) Nevinsky et al. (1974); (20) Lemaire et al. (1967); (21) Calendar & Berg (1966); (22) Mitra & Mehler (1967); (23) Peterson & Fowden (1965); (24) Papas & Mehler (1970); (25) Fowden & Richmond (1963); (26) Atherly & Bell (1964); (27) Conway et al. (1962); (28) Anderson & Fowden (1970a); (29) Santi & Danenberg (1971); (30) Smith & Fowden (1968); (31) this work; (32) Porter et al. (1977); (33) Lodeman et al. (1977); (34) Shirota et al. (1977).

valine system evolved solely in this enzyme or is it a general phenomenon in this class of enzymes? The occurrence of a similar activity in valyl-tRNA synthetase for the prevention of threonylation of $tRNA^{Val}$ has already been established (Fersht & Kaethner, 1976; Igloi et al., 1977) and we would now like to extend this concept to other, novel, misactivations.

## Experimental Section

*Materials.* $tRNA^{Phe}$-C-C and $tRNA^{Val}$-C-C were isolated by the procedure of Schneider et al. (1972) from unfractionated baker's yeast (Boehringer, Mannheim, West Germany). Incorporation of the appropriate terminal nucleotide (AMP or 3′-deoxy-3′-aminoadenosine monophosphate) was performed using tRNA-nucleotidyl transferase (EC 2.7.7.25) as described (Sprinzl & Cramer, 1978). Valyl-, phenylalanyl-, and tyrosyl-tRNA synthetases (EC 6.1.1.9, 6.1.1.20, and 6.1.1.1, respectively) were purified from baker's yeast by affinity elution (von der Haar, 1973; Faulhammer & Cramer, 1977). [$^{14}$C]ATP (approximately 45 Ci/mol) and [$^{14}$C]amino acids (of Stanstar Grade at 50 Ci/mol) were obtained from Schwarz Bioresearch (Orangeburg, N.Y.). [$^{32}$P]Pyrophosphate was the product of the Radiochemical Centre (Amersham, U.K.). 1-Aminocyclopentane-1-carboxylic acid (cycloleucine) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.) as was L-norvaline. DL-Homoserine and 1-aminocyclopropane-1-carboxylic acid (cyclopropylglycine) were obtained from Calbiochem (San Diego, Calif.). L-Peni-

TABLE II: Substrate Properties of Some Valine Analogues for Valyl-tRNA Synthetase from Yeast.

| analogue | ATP-$PP_i$ exchange $K_m$ (mM) | ATP-$PP_i$ exchange $V_{max}$ (% Val) | ATP hydrolysis: fraction of ATP[d] (%) | amount of $tRNA^{Val}$-C-C-A(3′$NH_2$)[e] (%) |
|---|---|---|---|---|
| L-α-aminobutyric acid | 2.8 | 20.0 | 100 | 97[a] |
| cycloleucine | 10.0 | 10.0 | 44 | 90 |
| cyclopropylglycine | nd[b] | nd | 4 | 15 |
| DL-homoserine | 0.9[c] | 4.0 | 9 | 34 |
| L-*O*-methylthreonine | 10.0 | 18.0 | 2 | 89[a] |
| L-norvaline | 33.0 | 4.0 | 12 | 90 |
| L-penicillamine | 1.2 | 2.0 | 5 | 11 |

[a] 0.2 mM instead of 1 mM. [b] nd, not determined. [c] Based on the concentration of the L isomer. [d] Fraction of ATP converted to AMP in 20 min with 1 mM analogue. [e] Amount of $tRNA^{Val}$-C-C-A(3′$NH_2$) aminoacylated in 60 min with 1 mM analogue.

cillamine was from Serva (Heidelberg, West Germany). All other amino acids were purchased from Sigma (St. Louis, Mo.). Other chemicals were commercially available analytical grade products.

*ATP-$PP_i$ Exchange.* The exchange of [$^{32}$P]pyrophosphate into ATP catalyzed by an aminoacyl-tRNA synthetase was monitored as described (Simlot & Pfaender, 1973; von der Haar & Gaertner, 1975).

$$E^{Xxx} + Yyy + ATP \rightleftharpoons E^{Xxx}\cdot Yyy\text{-}AMP + PP_i \quad (1)$$

The incubation mixture contained 150 mM Tris-HCl (pH 7.65), 200 mM KCl, 5 mM $MgSO_4$, 1.5 mM ATP, 1.5 mM [$^{32}$P]$PP_i$ (at a specific activity of approximately 3000 cpm per nmol), and varying concentrations of amino acid in a total volume of 0.1 mL. The reaction was initiated by the addition of 30–50 μg of synthetase. The reaction was maintained at 37 °C.

*Aminoacylation of tRNA.* The aminoacylation of unfractionated tRNA was performed at 37 °C in 0.1 mL of solution containing 150 mM Tris-HCl (pH 7.65), 50 mM KCl, 10 mM $MgSO_4$, 2 mM ATP, 0.02 mM $^{14}$C-labeled amino acid and 1 mg of unfractionated tRNA.

$$E^{Xxx} + Yyy + ATP + tRNA\text{-}C\text{-}C\text{-}A \rightleftharpoons E^{Xxx} + AMP + PP_i + Yyy\text{-}tRNA\text{-}C\text{-}C\text{-}A \quad (2)$$

The reaction was started by the addition of 30–50 μg of synthetase. The incorporation of $^{14}$C-labeled amino acid into tRNA was measured as described (von der Haar & Gaertner, 1975). The aminoacylation of tRNA-C-C-A(3′$NH_2$)[1] was carried out under the conditions described above but using 0.05 mM $^{14}$C-labeled amino acid and 4.5 μM tRNA-C-C-A(3′-$NH_2$). The detection of the transfer of nonradioactive amino acid to tRNA-C-C-A(3′$NH_2$) by the method of back-titration (eq 3) has been described (Igloi et al., 1977).

$$E^{Xxx} + Yyy + ATP + tRNA^{Xxx}\text{-}C\text{-}C\text{-}A(\text{'}NH_2) \xrightarrow{\text{preincubation}}$$

$$E^{Xxx} + Yyy\text{-}tRNA^{Xxx}\text{-}C\text{-}C\text{-}A(\text{'}NH_2) + AMP + PP_i$$

quantitation of nonacylated tRNA | [$^{14}$C]Xxx + ATP

$$E^{Xxx} + [^{14}C]Xxx\text{-}tRNA^{Xxx}\text{-}C\text{-}C\text{-}A(\text{'}NH_2) + AMP + PP_i \quad (3)$$

[1] Abbreviations used: $tRNA^{Xxx}$, $tRNA^{Xxx}$-C-C-A (tRNA specific for amino acid Xxx); Yyy-$tRNA^{Xxx}$-C-C-A, tRNA specific for amino acid Xxx and aminoacylated with amino acid Yyy; $tRNA^{Phe}$-C-C-A(3′$NH_2$), $tRNA^{Phe}$ with terminal 3′-amino-3′-deoxyadenosine instead of adenosine; $tRNA^{Phe}$-C-C-A(′$NH_2$), $tRNA^{Phe}$ with terminal 3′-amino-3′-deoxy- or 2′-amino-2′-deoxyadenosine instead of adenosine.

*ATP Hydrolysis during Aminoacylation.* Production of AMP under aminoacylating conditions (eq 4) was followed by the method of von der Haar & Cramer (1976).

$$E^{Xxx} + Yyy + ATP + tRNA^{Xxx}\text{-}C\text{-}C\text{-}A \rightleftharpoons E^{Xxx}\cdot Yyy\text{-}tRNA^{Xxx}\text{-}C\text{-}C\text{-}A + AMP + PP_i \quad (4)$$

## Results

*Valyl-tRNA Synthetase and Amino Acid Analogues.* It has previously been found (Igloi et al., 1977) that valyl-tRNA synthetase (yeast) will activate (and transfer) both L-α-aminobutyric acid and L-*O*-methylthreonine. Other reports of the utilization of amino acid analogues by the valine activating enzyme from different sources have suggested a relative lack of specificity in the recognition of structural features of the amino acid by the synthetase (Table I). The yeast enzyme, which has so far not been the subject of extensive amino acid analogue studies, proves to be no exception in this respect. Table II shows that valyl-tRNA synthetase (yeast) will promote ATP-$PP_i$ exchange with a wide variety of structurally dissimilar amino acid analogues. These analogues, with the exception of DL-homoserine, had previously been found to be suitable substrates for the *E. coli,* rat liver, and/or *Aesculus californica* enzymes (Table I) with kinetic constants (where determined) of magnitude similar to those in Table II. This demonstrates again that neither branching at the β carbon (L-α-aminobutyric acid, L-norvaline) nor a solely alkane side chain (L-*O*-methylthreonine, DL-homoserine) are required for activation, although the L configuration is essential since neither D-norvaline nor D-alloisoleucine was activated. Even some extreme structural features such as a trisubstituted β carbon (L-penicillamine) and cyclic side chains (cycloleucine, cyclopropylglycine) are tolerated.

Of the compounds tested in this work, previous studies had demonstrated the transfer of activated L-α-aminobutyric acid and L-*O*-methylthreonine to $tRNA^{Val}$ (Igloi et al., 1977) and Lodemann et al. (1977) had observed L-penicillamine esterified to $tRNA^{Val}$ in the rat liver system. Some other valine analogues have also been found to be transferred to tRNA (L-α-amino-β-chlorobutyric acid in *E. coli,* Freundlich, 1967; cyclobutylglycine in *E. coli,* Porter et al., 1977). In the yeast system we have established that all those analogues which are activated are probably transferable to $tRNA^{Val}$. This was deduced from the fact that all the analogues take part in the ATP hydrolyzing reaction (Table II) but at a rate which is dependent on the structure of the analogue considered. Furthermore, aminoacylation of $tRNA^{Val}$-C-C-A(3′$NH_2$), as determined

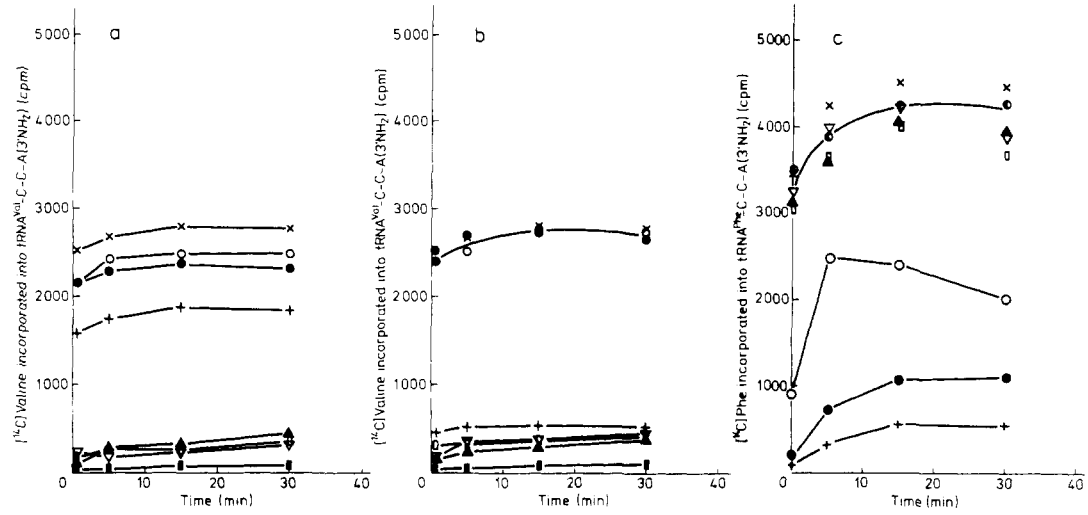



FIGURE 1: Determination of transfer of amino acids by valyl- and phenylalanyl-tRNA synthetase to $tRNA^{Val}$-C-C-A(3′$NH_2$) and $tRNA^{Phe}$-C-C-A(3′$NH_2$), respectively, by the method of back-titration. Amino acid, 1 mM (except for L-*O*-methylthreonine and L-α-aminobutyric acid, 0.2 mM), was incubated at 37 °C for 60 min in 150 mM Tris-HCl (pH 7.65), 50 mM KCl, 10 mM $MgSO_4$, 2 mM ATP, 5 μM $tRNA^{Val}$-C-C-A(3′$NH_2$) or $tRNA^{Phe}$-C-C-A(3′$NH_2$), and 8.6 μg of valyl-tRNA synthetase or 10.5 μg of phenylalanyl-tRNA synthetase in a total volume of 0.1 mL. The amount of nonaminoacylated tRNA was determined by the formation of [$^{14}$C]Val-$tRNA^{Val}$-C-C-A(3′$NH_2$) (a and b) or [$^{14}$C]Phe-$tRNA^{Phe}$-C-C-A(3′$NH_2$) (c) after the addition of 10 μL of a solution containing 0.5 mM [$^{14}$C]valine or phenylalanine and 5 mM ATP. The control reaction was carried out in the absence of added analogue or amino acid during the preincubation period. (a) Transfer of valine analogues by valyl-tRNA synthetase. (X—X) Control; (○—○) L-penicillamine; (●—●) cyclopropylglycine; (+—+) DL-homoserine; (▽—▽) cycloleucine; (□—□) L-norvaline; (▲—▲) L-*O*-methylthreonine; (■—■) L-α-aminobutyric acid. (b) Transfer of naturally occurring amino acids by valyl-tRNA synthetase. The concentration of threonine used in the preincubation was 0.2 mM and the controls included samples preincubated with the nonsubstrates methionine and leucine. (X—X) Control; (●—●) leucine; (○—○) methionine; (+—+) serine; (□—□) cysteine; (▽—▽) isoleucine; (▲—▲) alanine; (■—■) threonine. (c) Transfer of naturally occurring amino acids by phenylalanyl-tRNA synthetase. (X—X) Control; (◐—◐) glycine; (□—□) valine; (▽—▽) isoleucine; (▲—▲) alanine; (○—○) methionine; (●—●) leucine; (+—+) tyrosine.

TABLE III: Substrate Properties of Some Naturally Occurring Amino Acids for tRNA Synthetases from Yeast.

| amino acid | ATP-$PP_i$ exchange $K_m$ (mM) | ATP-$PP_i$ exchange $V_{max}$ (%)[h] | ATP hydrolysis[g] (%) | aminoacylation of unfractionated tRNA[d] (%) | amount of $tRNA^{Xxx}$-C-C-A(3′$NH_2$) aminoacylated[e] |
|---|---|---|---|---|---|
| | | | Valyl-tRNA Synthetase | | |
| Val | 0.17 | 100.0 | 25[b] | 100 | 100 |
| Cys | 3.8 | 47.6 | 73 | <1 | 87 |
| Ser | 1.5 | 4.4 | 8 | <1 | 81 |
| Ile | 7.1 | 57.2 | 23 | <1 | 86 |
| Ala | 33.0 | 5.2 | 9[a] ($K_m$ 7.7 mM) | <1 | 89 |
| Thr | 2.9 | 40.0 | 46[b] ($K_m$ 0.02 mM) | <1 | 96[c] |
| | | | Phenylalanyl-tRNA Synthetase | | |
| Phe | 0.035 | 100.0 | 50[f] | 100 | 100 |
| Tyr | 1.5 | 53.0 | 32 | <1 | 87 |
| Met | 0.23 | 7.9 | 16 | <1 | 55 |
| Leu | 0.19 | 2.1 | 2.5 | <1 | 75 |

[a] 1 mM amino acid. [b] 1 mM amino acid, from Igloi et al., 1977. [c] 0.2 mM amino acid, from Igloi et al., 1977. [d] Aminoacylation of unfractioned tRNA by appropriate tRNA synthetase (valyl-tRNA synthetase and phenylalanyl-tRNA synthetase, and units are in percent valine and phenylalanine, respectively). [e] Amount of $tRNA^{Val}$-C-C-A(3′$NH_2$) or $tRNA^{Phe}$-C-C-A(3′$NH_2$) aminoacylated in 60 min with 1 mM amino acid. [f] 1 mM amino acid; from von der Haar & Cramer, 1976. [g] Fraction of ATP converted to AMP in 20 min with 5 mM amino acid. [h] Percent valine and phenylalanine, respectively.

by the method of back-titration, is observed in each case (Figure 1).

*Valyl-tRNA Synthetase and Natural Amino Acids.* The ability of valyl-tRNA synthetase (yeast) to activate naturally occurring amino acids was investigated. The results summarized in Table III show that this enzyme will, in addition to threonine, misactivate alanine, cysteine, isoleucine, and serine. The evidence to support this novel finding has been obtained by a rigorous investigation of the behavior of these amino acids in the various steps of aminoacylation.

ATP-$PP_i$ exchange catalyzed by valyl-tRNA synthetase is stimulated by these five amino acids (Figure 2). The kinetic constants in each case show an increased $K_m$ and a decreased $V_{max}$ compared with valine but the relative drop in the activation efficiency depends on the individual amino acid considered, cysteine, isoleucine, and threonine being the most effectively misactivated. Leucine and methionine which also promoted the ATP-$PP_i$ exchange were found to be aminoacylated by valyl-tRNA synthetase to unfractionated tRNA but were not accepted by purified $tRNA^{Val}$-C-C-A(3′$NH_2$) (Figure 1). Their ATP-$PP_i$ exchange effect must therefore be considered to be due to trace impurities of leucyl-tRNA synthetase and/or methionyl-tRNA synthetase in our preparation of valyl-tRNA synthetase. Aminoacylation of unfractionated

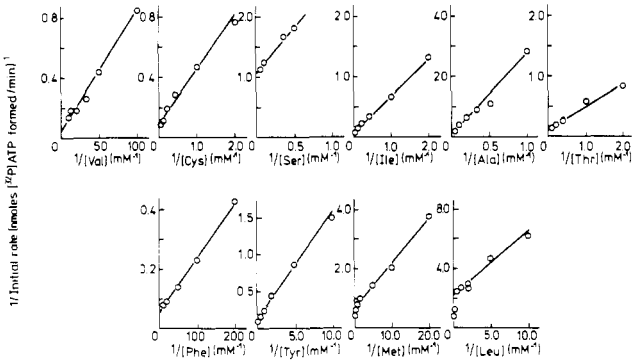


FIGURE 2: Lineweaver-Burk plots for the activation of naturally occurring amino acids by valyl-tRNA synthetase (above) and phenylalanyl-tRNA synthetase (below). The curves were obtained as described in Materials and Methods by the ATP-$PP_i$ exchange assay. Rates refer to 9 $\mu$g of valyl-tRNA synthetase and 5 $\mu$g of phenylalanyl-tRNA synthetase per aliquot. The kinetic constants derived from these plots are summarized in Table III.

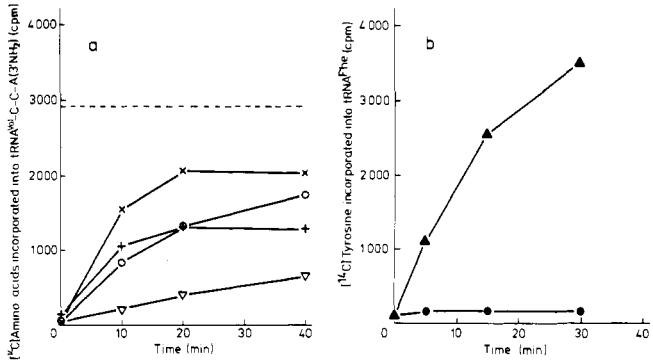


FIGURE 3: (a) Aminoacylation of $tRNA^{Val}$-C-C-A(3′$NH_2$) with $^{14}C$-labeled amino acids catalyzed by valyl-tRNA synthetase. The reaction was carried out as described in Materials and Methods. (- - -) Theoretical 100% aminoacylation level; (X—X) alanine; (O—O) isoleucine; (+—+) cysteine; (▽—▽) serine. (b) Aminoacylation of $tRNA^{Phe}$-C-C-A (●—●) and $tRNA^{Phe}$-C-C-A(3′$NH_2$) (▲—▲) with [$^{14}C$]tyrosine catalyzed by phenylalanyl-tRNA synthetase, using 5.7 $\mu$M $tRNA^{Phe}$-C-C-A(3′$NH_2$), as described in Materials and Methods.

tRNA by valyl-tRNA synthetase with alanine, cysteine, isoleucine, serine, or threonine resulted in an esterification of less than 1% compared with valine. $tRNA^{Val}$-C-C-A(3′$NH_2$), on the other hand, could be almost fully aminoacylated by each of the misactivated amino acids (Figure 1), although, as shown by the use of $^{14}C$-labeled amino acids (Figure 3), the rate of aminoacyl-$tRNA^{Val}$-C-C-A(3′$NH_2$) formation varied from compound to compound and was not directly predictable from the kinetic constants in the ATP-$PP_i$ exchange. The fact that transfer of misactivated amino acids to native tRNA also takes place is inferred from the nonstoichiometric ATP hydrolysis during aminoacylation of $tRNA^{Val}$-C-C-A with valyl-tRNA synthetase misactivated amino acids. The control, with a nonactivated amino acid (methionine or leucine), shows only a trace of this activity and this may be due to the enzymatic impurities mentioned above (Figure 4).

*Phenylalanyl-tRNA Synthetase and Natural Amino Acids.* Phenylalanyl-tRNA synthetase from various sources has been studied in detail with respect to its amino acid specificity (Table I). The wide variety of analogues which are accepted by this enzyme, together with the efficient hydrolytic capacity of the yeast enzyme (von der Haar & Cramer, 1976) was considered to be an indication that lack of specificity for natural amino acids may also occur in this case. Phenylalanyl-tRNA synthetase (yeast) for which the analogues in Table I have not been tested was examined for its ability to misactivate natural amino acids.

Of the 14 nonpolar or structurally similar amino acids tested, significant ATP-$PP_i$ exchange was obtained with leucine, methionine and, in particular, with tyrosine (Table III and Figure 2). These three amino acids were also transferred to $tRNA^{Phe}$-C-C-A(3′$NH_2$) by phenylalanyl-tRNA synthetase (Figures 1 and 3) but were not detected as stable aminoacyl-tRNA-C-C-A esters when tested with unfractionated tRNA (Table III). Further conclusive evidence for the misactivating capacity of phenylalanyl-tRNA synthetase (yeast) comes from the ATP splitting reaction during aminoacylation of $tRNA^{Phe}$-C-C-A, stimulated by leucine, methionine, and tyrosine but which is not observed when isoleucine is used as a control (Figure 4).

*Tyrosyl-tRNA Synthetase and Natural Amino Acids.* The 15 amino acids tested in the ATP-$PP_i$ exchange reaction catalyzed by tyrosyl-tRNA synthetase (yeast) under conditions where tyrosine itself stimulates rapid isotopic exchange all showed less than 1% of the exchange rate of tyrosine and were

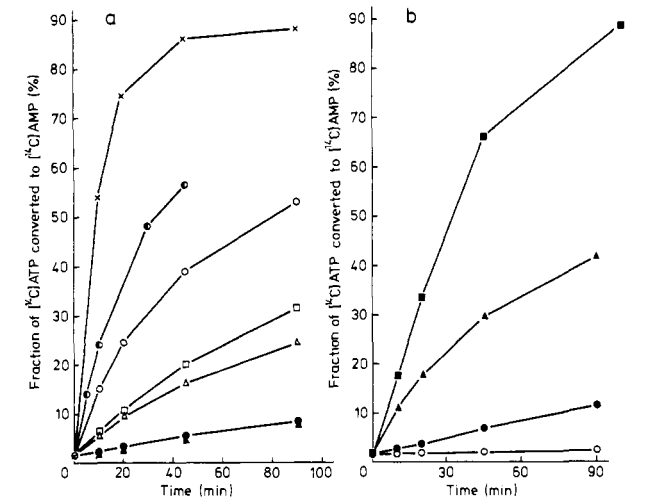


FIGURE 4: AMP formation by ATP hydrolysis during the aminoacylation of $tRNA^{Val}$-C-C-A by valyl-tRNA synthetase (a) and $tRNA^{Phe}$-C-C-A by phenylalanyl-tRNA synthetase (b) with various naturally occurring amino acids. The incubation, at 37 °C, consisted of 150 mM Tris-HCl (pH 7.65), 150 mM KCl, 10 mM $MgSO_4$, 5 mM amino acid, 0.5 mM [$^{14}C$]-ATP, 4.5 $\mu$M $tRNA^{Val}$-C-C-A or 5.4 $\mu$M $tRNA^{Phe}$-C-C-A in a total volume of 50 $\mu$L. The reaction was initiated by the addition of 17 $\mu$g of valyl-tRNA synthetase or 10.5 $\mu$g of phenylalanyl-tRNA synthetase. The estimation of [$^{14}C$]AMP formation was according to von der Haar & Cramer (1976). (X—X) Cysteine; (O—O) isoleucine; (▲—▲) serine; (●—●) leucine; (▲—▲) methionine; (□—□) alanine; (◐—◐) threonine (1 mM in incubation; from Igloi et al., 1977); (■—■) tyrosine.

therefore not considered to be activated by tyrosyl-tRNA synthetase.

*Competition between Correct and Incorrect Amino Acids for tRNA.* In order to evaluate misactivation in terms of the in vivo reaction, the competition between correct and incorrect amino acids for tRNA-C-C-A (3′$NH_2$) was considered.

Figure 5 shows the effect of the addition of nonradioactive valine to the aminoacylation of $tRNA^{Val}$-C-C-A(3′$NH_2$) with [$^{14}C$]threonine. At a 200 $\mu$M concentration of threonine, 3 $\mu$M valine is sufficient to cause a 50% drop in the extent of Thr-$tRNA^{Val}$-C-C-A(3′$NH_2$). A similar concentration of nonradioactive phenylalanine will inhibit [$^{14}C$]tyrosine incorporation into $tRNA^{Phe}$-C-C-A(3′$NH_2$) by 50% (Figure 5). Interestingly, though, nonradioactive tyrosine at a concentration in the mM range will compete with [$^{14}C$]phenylalanine transfer to $tRNA^{Phe}$-C-C-A(3′$NH_2$) when phenylalanine is

TABLE IV: Naturally Occurring Amino Acids Misactivated by Aminoacyl-tRNA Synthetases.

| enzyme specific for | amino acid | method of determination | ref |
|---|---|---|---|
| Ile (*E. coli* B) | Val | $PP_i$ exchange | Baldwin & Berg, 1966; Kondo & Woese, 1969 |
| Ile (*E. coli* K12) | Val, Leu | adenylate formation | Bergmann et al., 1961; Loftfield & Eigner, 1966; Owens & Bell, 1970 |
| Val (*E. coli*) | Thr, Ala, Ile | | |
| Val (yeast) | Thr, Ala, Ser, Cys, Ile | $PP_i$ exchange | this work |
| Phe (yeast) | Met, Leu, Tyr | modified tRNAs (see text) | this work |
| Leu (*Aesculus hippocastanum*) | Ile | $PP_i$ exchange | Anderson & Fowden, 1970b |

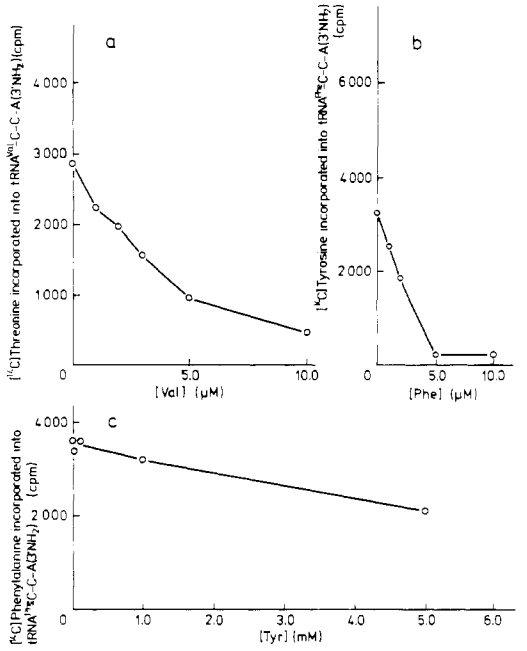



FIGURE 5: (a) Competition between [$^{14}$C]threonine and valine for aminoacylation of tRNA$^{Val}$-C-C-A(3′NH$_2$) by valyl-tRNA synthetase. The incubation, at 37 °C, contained 150 mM Tris-HCl (pH 7.65), 50 mM KCl, 10 mM $MgSO_4$, 2 mM ATP, 5 μM tRNA$^{Val}$-C-C-A(3′NH$_2$), 0.2 mM [$^{14}$C]threonine, and an increasing amount of valine. Valyl-tRNA synthetase (17.5 μg) was used in this reaction. (b) Competition between [$^{14}$C]tyrosine and phenylalanine for aminoacylation of tRNA$^{Phe}$-C-C-A(3′NH$_2$). The incubation was as in a, using 5.7 μM tRNA$^{Phe}$-C-C-A(3′NH$_2$), 0.2 mM [$^{14}$C]tyrosine, and an increasing amount of phenylalanine. The reaction was started by the addition of 10.5 μg of phenylalanyl-tRNA synthetase. (c) Competition between [$^{14}$C]phenylalanine and tyrosine for aminoacylation of tRNA$^{Phe}$-C-C-A(3′NH$_2$) by phenylalanyl-tRNA synthetase. The reaction was carried out as in b, using 0.1 mM [$^{14}$C]phenylalanine and an increasing amount of tyrosine. The aminoacylation was achieved using 0.1 μg of phenylalanyl-tRNA synthetase.

at $K_{diss}$ saturating concentrations ($K_{diss}$ = 5–10 μM) (Figure 5).

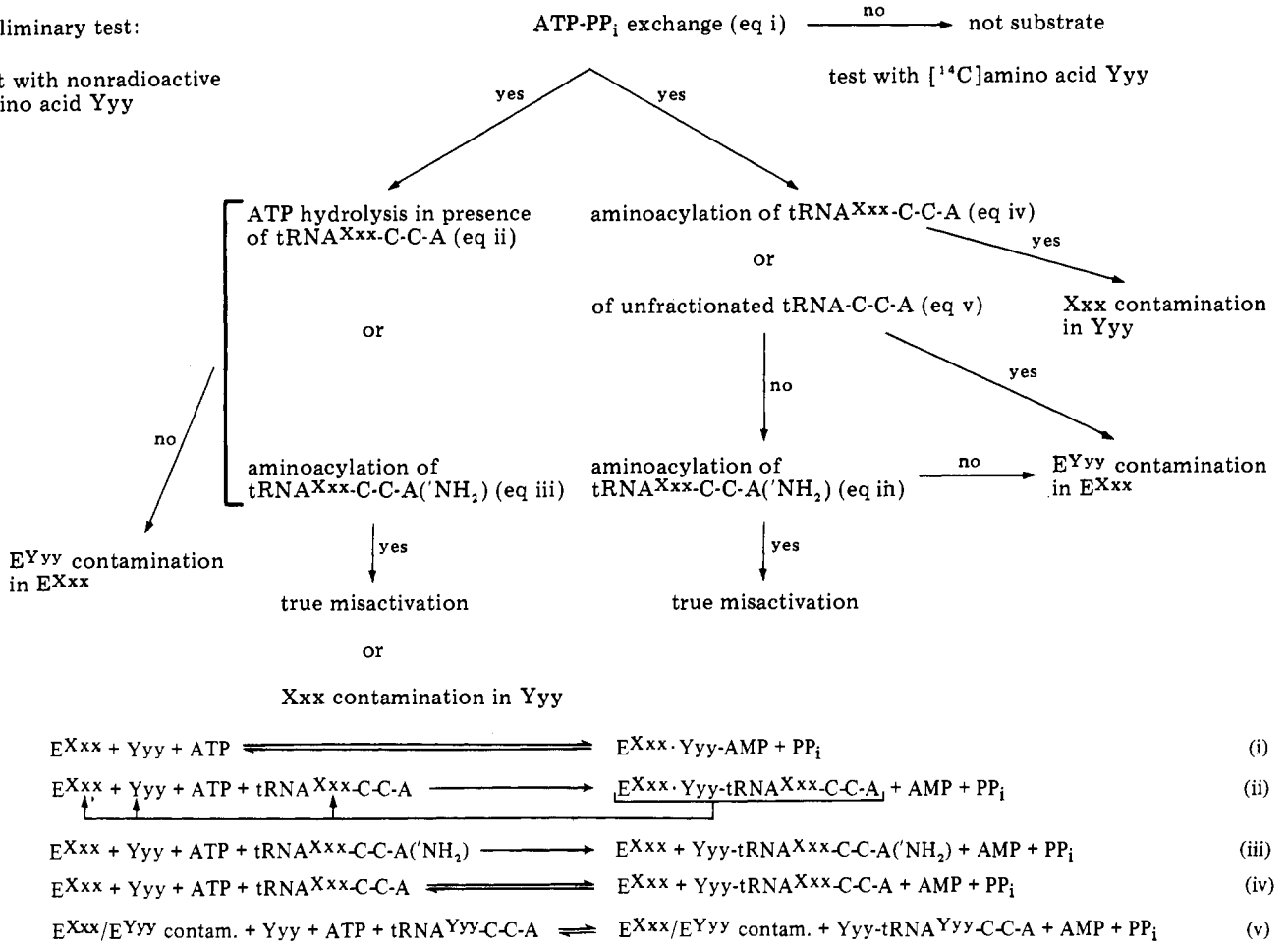
## Discussion

Despite ones belief in the high specificity of the synthetases for their amino acids, there is in some of the cases which have been examined a remarkable degree of nonspecificity for the activation of amino acid analogues whose structure may bear only a slight resemblance to the real substrate (Table I). Apart from the examples given in the table there are many more analogues which are efficiently bound but which are not activated by the synthetases (e.g., Flossdorf et al., 1976). It would appear, therefore, that there may only be a limited structural barrier to prevent the binding and misactivation of natural amino acids. Undoubtedly, there are kinetic consequences to the misactivation of an amino acid which can cause a sharp increase in the $K_m$ and a reduction in the $V_{max}$ during ATP–$PP_i$ exchange but, as has been shown in the isoleucyl-tRNA synthetase and valyl-tRNA synthetase cases (von der Haar & Cramer, 1976; Igloi et al., 1977), this type of differentiation between cognate and noncognate substrates may not be sufficient to bring about the required specificity. Examination of the literature reveals observations on synthetase specificity which have suggested that the misactivation of other natural amino acids may be taking place (Table IV). These marginal results have usually remained uninterpreted or have been treated as impurity induced artefacts.

The existence of misactivation can be established in a preliminary fashion by the ATP–$PP_i$ exchange reaction (eq i of Scheme I). The absence of such an exchange may be indicative of a nonsubstrate but could also occur in those cases where the conditions for exchange are unfavorable, e.g., absence of tRNA in the glutaminyl-tRNA synthetase system (Söll & Schimmel, 1974) or in the spermidine catalyzed Tyr-tRNA formation in the absence of $Mg^{2+}$ (Chousterman & Chapeville, 1973). An exchange of this sort is, however, by no means definitive evidence that the amino acid in question can be regarded as a substrate for the given aminoacyl-tRNA synthetase. Problems of contamination in either the enzyme preparation or in the amino acid, which would give false information in the above test, can be eliminated by a more rigorous examination of some other synthetase catalyzed steps according to Scheme I.

The homogeneity of the enzyme sample can be established by an investigation of the transfer of the activated amino acid. The experiment involves the reaction between $E^{Xxx}$, [$^{14}$C]Yyy, and an unfractionated tRNA mixture containing acceptors for all the amino acids (including Yyy). If $E^{Xxx}$ is contaminated with $E^{Yyy}$, [$^{14}$C]Yyy will become detectable in the acid-precipitable tRNA (see with valyl-tRNA synthetase). Since under normal conditions contaminating $E^{Yyy}$ is unlikely to recognize tRNA$^{Xxx}$, any aminoacylation of tRNA$^{Xxx}$ must be due to $E^{Xxx}$. The aminoacylation of tRNA$^{Xxx}$ with Yyy can be confirmed according to Scheme I. Two procedures are available for monitoring the transfer of nonradioactive amino acid to tRNA. Since the natural incorporation of incorrect amino acids into proteins is very low (e.g., Loftfield & Vanderjagt, 1972), any misacylation of tRNA must be corrected by enzymatic hydrolysis of the assumed intermediate Yyy-tRNA$^{Xxx}$ and a continuous nonstoichiometric utilization of ATP would be expected (eq ii of Scheme I). Such ATP splitting during aminoacylation of tRNA$^{Xxx}$-C-C-A is probably indicative of transfer of the incorrect amino acid (see below). A more direct determination of the misacylation of tRNA

SCHEME I: Functional Tests for the Misactivation of Natural Amino Acids.




$$E^{Xxx} + Yyy + ATP \rightleftharpoons E^{Xxx}\cdot Yyy\text{-}AMP + PP_i \quad (i)$$

$$E^{Xxx} + Yyy + ATP + tRNA^{Xxx}\text{-}C\text{-}C\text{-}A \longrightarrow E^{Xxx}\cdot Yyy\text{-}tRNA^{Xxx}\text{-}C\text{-}C\text{-}A + AMP + PP_i \quad (ii)$$

$$E^{Xxx} + Yyy + ATP + tRNA^{Xxx}\text{-}C\text{-}C\text{-}A('NH_2) \longrightarrow E^{Xxx} + Yyy\text{-}tRNA^{Xxx}\text{-}C\text{-}C\text{-}A('NH_2) + AMP + PP_i \quad (iii)$$

$$E^{Xxx} + Yyy + ATP + tRNA^{Xxx}\text{-}C\text{-}C\text{-}A \rightleftharpoons E^{Xxx} + Yyy\text{-}tRNA^{Xxx}\text{-}C\text{-}C\text{-}A + AMP + PP_i \quad (iv)$$

$$E^{Xxx}/E^{Yyy}\ \text{contam.} + Yyy + ATP + tRNA^{Yyy}\text{-}C\text{-}C\text{-}A \rightleftharpoons E^{Xxx}/E^{Yyy}\ \text{contam.} + Yyy\text{-}tRNA^{Yyy}\text{-}C\text{-}C\text{-}A + AMP + PP_i \quad (v)$$

(without resorting to the fast reaction method of Fersht (Fersht & Kaethner, 1976)) can be obtained by observing the stable amide formation between activated Yyy and $tRNA^{Xxx}$-C-C-A('$NH_2$) by the method of back-titration (Igloi et al., 1977; eq iii of Scheme I; see also eq 3).

Confirmation of the above interpretation and elimination of the possibility that contaminating Xxx is the cause of the observed phenomena can be obtained using radioactive amino acids. One can conclude that the misactivation in question is not artefactual, if no $^{14}C$ label from Yyy becomes incorporated into $tRNA^{Xxx}$-C-C-A (since correction takes place) but $tRNA^{Xxx}$-C-C-A('$NH_2$) is aminoacylated (e.g., Figure 3b).

The sequence of reactions described here and summarized in Scheme I allow one to identify unequivocally the misactivation of naturally occurring amino acids.

The specificity of valyl-tRNA synthetase (*E. coli*) has previously been examined by a number of workers (Bergmann et al., 1961; Loftfield & Eigner, 1966; George & Meister, 1967; Yaniv & Gros, 1969; Owens & Bell, 1970). The results are generally comparable but one or two notable exceptions exist. Thus, both Loftfield & Eigner (1966) and Yaniv & Gros (1969) found sufficient isoleucine dependent ATP-$PP_i$ exchange to be able to obtain kinetic constants for the reaction. In the former case the $K_m$ was as low as 3 mM with a $V_{max}$ of 10% of that of valine, while in the latter work the $K_m$ was 100 mM with $V_{max}$ being 22%. Owens & Bell (1970) and Bergmann et al. (1961) found only trace activity with isoleucine while George & Meister (1967) observed a negligible extent of ATP-$PP_i$ exchange with isoleucine. Alanine, which was inactive in the system of George & Meister (1967), inhibited the valine dependent ATP-$PP_i$ exchange of Loftfield & Eigner (1966) ($K_i$ = 100 mM) but was itself found to be a substrate for valyl-tRNA synthetase by Owens & Bell (1970) ($K_m$ = 39 mM; $V_{max}$ = 3%).

The more detailed study of valyl-tRNA synthetase (yeast) which we report here again shows the trend for a relative lack of specificity in the activation of substrate analogues (Table II), and, in view of the current concepts on the fidelity in aminoacylation, a surprising, if weak, potential for utilizing several natural amino acids as substrates. Despite the low efficiency of the misactivation, the rigorous and extensive tests which we have developed rule out any possibility of artefactual interference with these results. Valyl-tRNA synthetase (yeast) will, therefore, in addition to threonine misactivate serine, cysteine, alanine, and isoleucine. The acceptance of isoleucine by valyl-tRNA synthetase is perhaps not surprising in view of the number of substrate analogues which are shared by both isoleucyl-tRNA synthetase and valyl-tRNA synthetase (Table I; alloisoleucine, norvaline, and *O*-methylthreonine) while the small amino acids serine, alanine, and cysteine might be expected to be accommodated in the structurally larger valine binding site.

In the case of phenylalanyl-tRNA synthetase (yeast) there is no direct evidence in the literature for the utilization of a second naturally occurring amino acid as a substrate; indeed, the phenylalanyl-tRNA synthetase (*E. coli*) is said not to activate tyrosine or leucine as determined by the rather insensitive hydroxamate assay (Conway et al., 1962). However, both the *E. coli* enzyme and the phenylalanyl-tRNA synthetase from several *Aesculus* species will accept a wide variety of substrate

analogues (Table I) including some open chain structures which may be very effectively activated. Some differences are seen in this respect between the *E. coli* and the *Aesculus* enzymes (e.g., methallylglycine and ethallylglycine are not activated by the phenylalanyl-tRNA synthetase (*E. coli*)) but of particular interest is one difference between the phenylalanyl-tRNA synthetase of *A. californica* and the enzyme of other *Aesculus* species (Anderson & Fowden, 1970a). Each of these enzymes will activate 2-amino-4-methylhex-4-enoic acid. However, in *A. californica* this compound occurs naturally and yet is not incorporated into its proteins. A mechanism must therefore exist which prevents the activated phenylalanine analogue from being included into the growing peptide chain. [This is different from the case of azetidine-2-carboxylic acid which is activated by prolyl-tRNA synthetase (*E. coli*) (Papas & Mehler, 1970) but is inert toward the prolyl-tRNA synthetase of various plant species (Peterson & Fowden, 1965) in which it occurs naturally]. This then is the first indication that the phenylalanyl-tRNA synthetase may have a proofreading capacity similar in nature to that found previously in isoleucyl-tRNA synthetase and valyl-tRNA synthetase (von der Haar & Cramer, 1976; Igloi et al., 1977). Our results with phenylalanyl-tRNA synthetase (yeast) confirm this suspicion. This enzyme will activate leucine, methionine, and tyrosine. It is, perhaps, of interest to note that leucine, methionine, and possibly tyrosine show a distinctive nonlinear behavior at high concentrations during the ATP–$PP_i$ exchange catalyzed by phenylalanyl-tRNA synthetase (Figure 2). This type of kinetic feature has been described as being due to allosteric effects and may be relevant to the current ideas on the interaction between binding sites on the synthetases (Mulvey & Fersht, 1977; Fayat et al., 1977; Jacques & Blanquet, 1977). This, however, was not investigated further.

As with the valine enzyme, some indirect evidence exists in the case of leucine which one would consider as being a consequence of this misactivation. Not only is methallylglycine also a substrate for leucyl-tRNA synthetase (Table I), but perhaps more importantly leucine has been found to inhibit $Phe\text{-}tRNA^{Phe}$ formation catalyzed by phenylalanyl-tRNA synthetase (*E. coli*) (Kondo, 1971).

What is the fate of the misactivated amino acids? The scheme of experiments outlined above for the definitive identification of misactivated amino acids also leads to results which contribute to the solution of this question. The misactivated amino acids are not isolable as stable aminoacyl-tRNA-C-C-A esters and yet two types of experiment show that the tRNA must play an active role in the overall reaction. Firstly, the ATP hydrolyzing reaction (Figure 4) which all misactivated amino acids support has been ascribed to a continuous acylation/deacylation cycle (von der Haar & Cramer, 1976; Igloi & Cramer, 1978) and the aminoacyl-tRNA-C-C-A is probably an intermediate in this pathway. Although the transient transfer of misactivated threonine to $tRNA^{Val}$-C-C-A by valyl-tRNA synthetase has been demonstrated directly (Fersht & Kaethner, 1976), Fersht has argued that the main pathway for correction in the isoleucyl-tRNA synthetase (*E. coli*) reaction is hydrolysis of Val-AMP rather than hydrolysis of $Val\text{-}tRNA^{Ile}$ (Fersht, 1977). The only experimental evidence for this statement is that hydrolysis of preformed $Val\text{-}tRNA^{Ile}$ by isoleucyl-tRNA synthetase is a factor of 15 too slow to account for the rate of AMP formation in the complete aminoacylation system. Unfortunately only very little experimental data are given regarding this important experiment. The data given by Fersht seem to reflect, however, the second-order rate of association of $Val\text{-}tRNA^{Ile}$ with isoleucyl-tRNA synthetase rather than the rate of hydrolysis of $E^{Ile}$·$Val\text{-}tRNA^{Ile}$.

With respect to the isoleucyl-tRNA synthetase from baker's yeast, a $tRNA^{Ile}$ induced hydrolysis of $E^{Ile}$·Val-AMP would have to be an extremely specific reaction. Only the native $tRNA^{Ile}$-C-C-A is able to induce this hydrolysis which is not even stimulated by the $tRNA^{Ile}$-C-C-2′dA (von der Haar & Cramer, 1976), which in all other aspects mimics the behavior of $tRNA^{Ile}$-C-C-A (von der Haar & Cramer, 1978). This shows that for a putative hydrolysis of $E^{Ile}$·Val-AMP induced by $tRNA^{Ile}$ both the hydroxyls of the 3′-terminal adenosine are essential. This necessity for the diol function is we feel best explained by a transient transfer of the misactivated amino acid.

Amino acids which are not activated do not promote ATP hydrolysis (Figure 4). It could be argued that the low rate of ATP hydrolysis in some cases and the corresponding slow correction process would not be sufficient to contribute to an in vivo hydrolysis of an incorrectly formed aminoacyl-tRNA. Such considerations, however, ignore the contribution of other kinetic pathways to the overall mechanism (e.g., dissociation of aminoacyl-tRNA from the enzyme) for which only limited data are available. Secondly, if the potentially labile ester bond of aminoacyl-tRNA is replaced by a stable amide link as in the aminoacylation of tRNA-C-C-A(′$NH_2$), the transfer of misactivated amino acids is observed (Figures 3 and 1).

Three out of the four synthetases which have been examined (isoleucyl-tRNA synthetase, valyl-tRNA synthetase, and phenylalanyl-tRNA synthetase) have been shown to lack absolute specificity in amino acid activation but in each case a correction process prior to release of the misacylated tRNA reduces the possibility of incorporation of errors into the protein. The fourth enzyme studied (Tyrosyl-tRNA synthetase) will activate only the correct amino acid. In view of the inability of tyrosyl-tRNA synthetase (yeast) to hydrolyze the aminoacyl-tRNA ester link (von der Haar & Cramer, 1976), this observation is in accordance with expectations. Specificity for this enzyme must occur at the initial amino acid binding stage since the later hydrolytic correction of errors cannot be brought about by this enzyme. Indeed, in those species where D-tyrosine is activated by tyrosyl-tRNA synthetase, a separate enzyme exists to promote the breakdown of D-Tyr-tRNA (Calendar & Berg, 1966).

How much significance do these misactivations have in vivo? It can be argued that the low efficiency (high $K_m$, low $V_{max}$) with which these reactions occur in vitro would render them insignificant in an vivo situation where competition with the true substrate would further reduce the misactivation rate. It should, however, be remembered that, in a typical yeast cell, the amino acid pool is large (Holden, 1962), and the concentration of free amino acids is in the mM range. If the $K_m$s of the misactivated amino acids in the ATP–$PP_i$ exchange reaction are reduced by the presence of tRNA as was found in the correction of threonine misactivation by valyl-tRNA synthetase (Igloi et al., 1977) and to a smaller extent in the correction of alanine misactivation by valyl-tRNA synthetase even noncognate amino acids would be expected to bind to synthetases to a certain extent (Tables II and III). The fact that noncognate amino acids can compete with cognate ones for transfer to tRNA is demonstrated by the effect of valine on the transfer of misactivated threonine to $tRNA^{Val}$-C-C-A(3′$NH_2$) and of tyrosine on the transfer of correctly activated phenylalanine to $tRNA^{Phe}$-C-C-A(3′$NH_2$) (Figure 5). In the latter case it is seen that at near saturating concentrations of phenylalanine ($K_{diss}$ = 5–10 $\mu$M), tyrosine at cellular concentrations (Holden, 1962) causes significant inhibition of transfer. One would anticipate that competition for the native tRNAs

would be similar. These tests are clearly susceptible to trace amino acid Xxx impurities in Yyy, but we have shown that in the case of phenylalanyl-tRNA synthetase activated [$^{14}$C]-tyrosine no radioactive material is incorporated into tRNA$^{Phe}$-C-C-A (Figure 3b), indicating the absence of [$^{14}$C]phenylalanine in [$^{14}$C]tyrosine. Similar experiments had established the absence of [$^{14}$C]valine in [$^{14}$C]threonine (Igloi et al., 1977).

The situation for the naturally occurring amino acids considered in this work parallels the behavior of the well-documented isoleucyl-tRNA synthetase/valine and valyl-tRNA synthetase/threonine misactivations (von der Haar & Cramer, 1976; Fersht & Kaethner, 1976; Igloi et al., 1977) and the fact that the capability to misactivate several amino acids exists implies that a chemical proofreading mechanism for increasing the specificity of aminoacylation is more widespread than has been supposed. Indeed, we would take the opposite point of view to that of Fayat et al. (1977) and suggest that it is not always the primary binding which defines the specificity and that misactivation is not a rare phenomenon. It might then be chemical proofreading, in the sense of a positive enzymatic hydrolysis of a misacylated aminoacyl-tRNA, which is one "key feature of the overall specificity of the aminoacylation reaction".

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# Isolation and Characterization of Polyadenylate-Containing RNA from *Bacillus brevis*†

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ABSTRACT: A substantial fraction (30–40%) of pulse-labeled RNA from exponentially growing cells of *Bacillus brevis* contains polyadenylate sequences, as measured by adsorption to oligo(dT)-cellulose. The weight-average length of poly(A) tracts obtained after digestion with pancreatic and $T_1$ ribonucleases is 60 nucleotide residues. Susceptibility to degradation by snake venom phosphodiesterase after ribonuclease degradation indicates that the poly(A) sequences are located near the 3′ ends of the RNA chains, but that in 40% of the material at least one internal pyrimidine nucleotide residue intervenes between the poly(A) tract and the 3′-hydroxyl terminus. These pyrimidine nucleotides consist of 65% cytidylate and 35% uridylate residues. In the remaining RNA chains, the poly(A) sequence is directly at the 3′-terminus, but the possibility cannot be excluded that a small fraction of this material may contain a 3′-hydroxyl terminal guanylate residue. The weight-average sedimentation coefficient of poly(A)-containing RNA is 12.5 S, corresponding to a polynucleotide chain length of 800–900 residues. This is in a size range expected for messenger RNA, a possibility which is also supported by the observation that pulse-labeled RNA has a considerably higher poly(A) content than long-term labeled RNA.

Since the discovery of poly(A) sequences in mRNA from eukaryotic cells (Darnell et al., 1971; Edmonds et al., 1971; Lee et al., 1971), much thought has been given to their possible function, but no clear answer has emerged. One possible approach to this question is to study the distribution of poly(A) sequences in nature, especially in simpler systems. In this connection, the presence of such RNA species was recently reported in several prokaryotic systems. Very low levels of poly(A) RNA have first been described in *Escherichia coli* (Nakazato et al., 1975), but somewhat higher levels (up to 15%) have been found under different growth conditions (Srinivasan et al., 1975). Relatively short poly(A) sequences (about 15 nucleotides) have also been found in *Caulobacter crescentus* mRNA (Ohta et al., 1975, 1978). In this paper, we report the occurrence of relatively high levels (30–40%) of poly(A)-containing molecules among the rapidly labeled RNA species of *Bacillus brevis* and describe their characterization.

## Experimental Procedure

*Materials.* These were obtained from the following sources: [5-$^3$H]uridine (25 Ci/mmol) and [2,8-$^3$H]adenosine (16.8 Ci/mmol) were from New England Nuclear; pancreatic ribonuclease A (Grade R, 2900 units/mg) and bacterial alkaline phosphatase (Grade BAPF, 55 units/mg) were from Worthington; $T_1$ ribonuclease (6500 units/mg) was from Calbiochem; snake venom phosphodiesterase (1.5 units/mg) was from Boehringer; proteinase K was from Beckman; poly(adenylic acid) was from P-L Biochemicals; poly(cytidylic acid) was from Miles; tRNA (*Escherichia coli*) was from Schwarz-Mann; oligo(dT)-cellulose (Type T2) was from Collaborative Research; PEI-cellulose[1] sheets were from Brinkmann; cellulose thin-layer sheets (with fluorescent indicator) were from Eastman; heparin was from Organon; and sucrose (density gradient grade) was from Schwarz-Mann. rRNA was isolated by phenol extraction from ribosomes of *B. brevis,* either unlabeled or labeled with [$^3$H]uridine. $(Ap)_{\overline{70}}$Up, containing [$^{14}$C]adenosine (0.1 $\mu$Ci/$\mu$mol) and [$^3$H]uridine (10 $\mu$Ci/$\mu$mol), was prepared as described previously (Sarkar and Paulus, 1975).

*Bacterial Growth and Labeling Procedures. Bacillus brevis* ATCC 8185 was grown in the medium of Hanson et al. (1964) at 37 °C on a rotary shaker. Growth was monitored with a Klett-Summerson photoelectric colorimeter using the No. 42 filter. During early exponential growth (100–110 Klett units), culture samples (1 mL) were treated with [5-$^3$H]uridine or [2,8-$^3$H]adenosine (generally 1–10 $\mu$Ci). After 30 s, the samples were quickly chilled to 4 °C and treated with 25 mM $NaN_3$. The cells were collected by centrifugation at 10 000*g* for 5 min and washed once with growth medium containing 25 mM $NaN_3$.

*Isolation of Poly(A)-Containing RNA.* The washed pulse-labeled cells were suspended in 0.38 mL of a solution containing 80 mM Tris-HCl, pH 9.0; 10 mM EDTA; 20 mM 1,10-phenanthroline; 0.5% sodium dodecyl sulfate; 0.2 mg/mL heparin; and 0.23 mg/mL proteinase K. The mixture was in-

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[1] Abbreviations used: PEI-cellulose, polyethylenimine-impregnated cellulose; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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