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Probing the principles of amino acid selection using the alanyl-tRNA synthetase from Escherichia coli

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ABSTRACT

The alanyl-tRNA synthetase from *Escherichia coli* activates cysteine, α-aminobutyrate and other non-cognate amino acids that are larger than alanine so slowly that no editing mechanism is required for error correction. Serine, however, is activated sufficiently rapidly that an editing mechanism is required to remove the products of misactivation. The distinction between the nominally isosteric trio of cysteine, α-aminobutyrate and serine by the activation site of the enzyme is attributed to the effect of small differences in size on steric repulsion, the C-O bond length being somewhat shorter than either the C-C or C-S and the van der Waals' radius of -O- being smaller than that of -S- or $-CH_2-$. The smaller amino acid glycine is also readily activated and its reaction products rapidly removed by hydrolytic editing.

INTRODUCTION

The mechanism of selection of amino acids by the aminoacyl-tRNA synthetases may be dissected into two components: *i*, the enzymes have evolved to bind the distinctive portions of their cognate substrates as tightly as possible and discriminate as much as possible against the binding of the non-cognate substrates; *ii*, where necessary, the enzymes have evolved an editing mechanism that removes the unwanted products of misactivation by hydrolysis. A simple hypothesis, the "double-sieve sorting mechanism", has been proposed to rationalise the wide variety of misactivations catalysed by these enzymes and predict the examples where editing may be necessary (1). Experiments on the valyl-tRNA synthetase have shown that just one additional methylene group in the side chain of a larger substrate (as in isoleucine) is sufficient to cause such unfavourable steric interactions with the enzyme that the larger substrates are activated so slowly as not to require subsequent editing. Smaller amino acids must, perforce, bind and be activated. Depending on the relative rate of activation of the smaller substrates, an editing reaction may be required to remove the products of their activation. The activation and editing sites of the aminoacyl-tRNA synthetase sort the whole range of

competing non-cognate substrates according to primarily size and chemical characteristics (1,2). Experiments on the activation of tyrosine by the phenylalanyl-tRNA synthetase, however, indicate that the presence of the additional oxygen atom in tyrosine is not sufficient for its steric exclusion at a tolerable level and that an editing mechanism is necessary for this larger substrate (3,4).

In the present study we examine the mechanism of sorting of amino acids by the alanyl-tRNA synthetase from *Escherichia coli*. This enzyme has the problem of rejecting three non-cognate amino acids which are slightly larger than alanine and are nominally isosteric: serine, cysteine and α-amino-butyric acid. The rate constants for activation of these and other non-cognate amino acids are measured using methods that eliminate the possibility of artefacts due to contamination by alanine. The trio of isosteric amino acids provide an exquisite probe for testing the rigidity of the protein structure in binding larger amino acids.

EXPERIMENTAL PROCEDURES

Materials. *E. coli* K12 strain KL 386 containing a hybrid pBR322 plasmid with an *ala*S insert (pSP101) was a generous gift from Dr. P.R. Schimmel. Alanyl tRNA synthetase was purified to apparent homogeneity according to Putney *et al* (5). Concentrations were determined by active site titration using nitrocellulose disc filtration at pH 6.0 or the ATP depletion method (6). Unfractionated tRNA from *E. coli*, provided by the Imperial College Pilot Plant, had an alanine acceptance of 60 pmol/A_{260}. The terminal adenosine residue of the tRNA was oxidised by periodate (7) to give material of an alanine acceptance of less than 0.2 pmol/A_{260}. ^{3}H-labelled L-α-aminobutyrate was prepared from the unlabelled material by the TRI labelling service of the Radiochemical Centre, Amersham. The crude material was purified by chromatography on SP-Sephadex C25 (8). The α[^{3}H]aminobutyrate was 97% radiochemically pure with a specific activity of 248 Ci/mol. α[1-^{14}C]Aminobutyrate was prepared by published procedures (8).

Radiochemicals were purchased from the Radiochemical Centre, Amersham, UK. Glycine, L-alanine and L-cysteine hydrochloride were obtained from BDH. ATP, yeast inorganic pyrophosphatase, unlabelled L-α-aminobutyrate, L-norvaline, L-norleucine and activated charcoal were obtained from Sigma. Amberlite SA-2 cation exchange paper was obtained from Whatman.

Methods. All kinetic measurements were performed unless otherwise stated at 25^{o}C in a standard buffer containing either 144 mM Tris (pH 7.8)

or 0.1 M Bis-Tris (pH 6.0), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.1 mM phenylmethane-sulphonyl fluoride. ATP-PP_i exchange in the presence of 2 mM ATP, 2 mM [^{22}P]pyrophosphate (specific activity 1 to 5 cpm/pmol) was measured by conventional procedures. Amino acid-dependent ATP-pyrophosphatase activity was measured from the release of [^{32}P]pyrophosphate from [γ-^{32}P]ATP (1). The reaction mixture (100 µl) contained 2 mM [γ-^{32}P]ATP (5 - 20 cpm/pmol), 1 U/mL yeast inorganic pyrophosphatase, 0.1 to 1.0 µM alanyl-tRNA synthetase and, in some cases, 1.76 µM $tRNA^{Ala}$ in the standard pH 7.8 buffer. Samples (18 µl) were withdrawn at intervals and quenched with 100 µl of 0.5N HCl containing 25 mg/ml KH_2PO_4 and 100 µl of charcoal. After centrifugation for 5 minutes with a benchtop centrifuge, duplicate samples of the supernatant were counted in a toluene-based scintillant.

Suppression of Formation of [^{14}C]Ala-$tRNA^{Ala}$ by Serine and α-Aminobutyrate. A solution (0.2 ml) containing $tRNA^{Ala}$ (12.5 µM), alanyl-tRNA synthetase (0.8 µM), ^{14}C-alanine (30 µM, 16.8 Ci/mol), inorganic pyrophosphatase (1 U/ml) and ATP (2 mM) in the standard pH 6.0 or 7.8 buffer was incubated at 25°. Samples (50 µl) were periodically quenched with ice cold 5% trichloroacetic acid and the precipitated aminoacyl-tRNA was collected on a glass fibre filter and assayed by scintillation counting. The procedure was repeated in the presence of added serine or α-aminobutyrate.

Scavenging Serine and α-Aminobutyrate to Remove Alanine. A reaction mixture (25 ml) containing serine or α-aminobutyrate (120 mM), ATP (10 mM), inorganic pyrophosphatase (5 U), alanyl-tRNA synthetase (0.3 µM) and hydroxylamine (1 M) in a standard pH 7.8 buffer was incubated at 37°. Samples (5 ml) were quenched at 24, 42 and 72 hr with glacial acetic acid (1.5 ml) and chromatographed on SP-Sephadex C-50 equilibrated with 4 M acetic acid. The amino acid was eluted with a gradient of acetic acid (4 M)-pyridine (0.04 M) and acetic acid (1 M)-pyridine (0.5 M). The resultant amino acid, after lyophilisation, was desalted on Amberlite IR-120 (1,9).

Rate and Extent of Formation of Aminoacyl-Hydroxamate. The reaction mixture (100 µl) contained NH_2OH.HCl (1 M) neutralized with NaOH, ^{14}C-alanine (30 µM), and alanyl-tRNA synthetase in the standard buffer. Samples (10 µl) were quenched at intervals with 12% trichloroacetic acid (10 µl) and the precipitated protein removed by centrifugation. The amino acid and the hydroxamate were separated as described by Loftfield and Eigner (10). Supernatant (10 µl) was applied to a strip of SA-2 paper (10 cm x 1 cm), 1.2 cm from one end. The paper was developed with 50 mM sodium phosphate, pH 7.0. The hydroxamate remained near the origin whereas the amino acid

moved with the solvent front. When the solvent moved to about 1 cm from the top, the paper was removed and dried under a heat lamp. Sections of paper 2.5 cm from the origin and 2.5 cm from the solvent front were cut and counted in a toluene-based scintillant. The amount of hydroxamate formed was expressed as a percentage of the total radioactivity. The procedure was repeated with ^{14}C-serine (5.72 mM) and ^{14}C-α-aminobutyrate (27.2 mM), the latter at 37^{o}C.

RESULTS

Activation of Non-Cognate Amino Acids. The data on the activation of amino acids by the alanyl-tRNA synthetase as measured by the pyrophosphate exchange reaction are summarised in Table 1. Amino acids with side chains larger than alanine (except serine, see below) are activated only very weakly, being efficiently excluded by steric hindrance from the active site. Glycine and serine, however, are misactivated at a significant rate with specificities relative to alanine of 1/240 and 1/500 respectively. This causes a problem of specificity in the selection of alanine by the enzyme. Glycine, being smaller than alanine is expected to be activated by the enzyme by the "double-sieve" hypothesis (1). The rate of misactivation of the larger molecule of serine is surprisingly high. As it has been previously noted that commercial

Table 1: Activation of L-Amino Acids Catalysed by the Alanyl-tRNA Synthetase from *E. coli*[a].

Amino Acid	k_{cat}(s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}M^{-1}$)	Relative Specificity[b]
Glycine	3.7	46	80	4×10^{-3}
Alanine	4.5	0.23	1.9×10^{4}	1
Serine[c]	4.3	112	38	2×10^{-3}
α-Aminobutyrate[c]		> 120	1.1	6×10^{-5}
Cysteine		> 10	1.6	8×10^{-5}
Norvaline	0.11	73	1.5	8×10^{-5}
Norleucine		> 70	0.55	3×10^{-5}

[a] Pyrophosphate exchange reaction at 25 ^{o}C, standard pH 7.78 buffer, 2 mM ATP, and 2 mM [^{32}P]pyrophosphate. [b] Relative values of k_{cat}/K_M.

[c] Samples scavenged for alanine by treatment with enzyme and hydroxylamine.

samples of amino acids sometimes contain residual trace impurities of other amino acids which are resistant to removal (1,9), it is necessary to establish that the pyrophosphate exchange activity observed with serine is not due to the presence of trace amounts of alanine or any other active amino acid. Three different approaches were used.

i, Scavenging of Amino Acids with Hydroxylamine and Enzyme. On treatment of serine with hydroxylamine ATP and the alanyl-tRNA synthetase for 72 hr to remove any alanine or other readily activated amino acid present as contaminant, the value of k_{cat}/K_M for the pyrophosphate exchange reaction dropped by only 40%. This was due to an increase in K_M, the value of k_{cat} remaining unchanged. Similarly, treating α-aminobutyrate for 72 hr removed only 40% of the pyrophosphate exchange activity. The residual activity in the pyrophosphate exchange reaction thus appears real.

ii, Determination of Alanine in the Non-Cognate Amino Acids by Isotopic Dilution (1). The upper limit of alanine present in the samples of serine was determined by measuring the extent of suppression of formation of [^{14}C]-Ala-tRNA^{Ala} in an aminoacylation mixture by the addition of serine. The (apparent) lowering of the extent of aminoacylation by serine is due to either the formation of Ser-tRNA^{Ala} or an impurity of alanine diluting the specific activity of [^{14}C]Ala. At 30 μM ^{14}C-alanine, the concentration of serine at which the extent of aminoacylation is lowered by 50% is 430 mM. Thus the upper limit of alanine in the sample of serine is $1/(1.4 \times 10^4)$, a value far too low to account for the efficient rate of pyrophosphate exchange.

iii, Activation of Serine, Glycine and α-Aminobutyrate Detected by Hydroxamate Formation. During the pyrophosphate exchange reaction, the amino acid is not consumed but acts only catalytically. To show that a substrate is genuinely active, it is desirable to use a reaction in which measurable quantities of products are formed and the substrate is consumed. Such a reaction is the formation of the aminoacyl hydroxamate as used by Loftfield and Eigner (10). In the presence of 1 M hydroxylamine and 30 μM ^{14}C-alanine in the standard pH 7.8 buffer, the hydroxamate is formed at an initial rate of 4×10^{-2} mol/mol-enzyme/s. Significant fractions of ^{14}C-serine, ^{14}C-glycine and ^{3}H-α-aminobutyrate were also converted to their hydroxamates: Serine (5.7 mM), > 30% conversion at $v_o = 8 \times 10^{-3}$ mol/mol-enzyme/s; glycine (25 mM), > 20%, 0.025 s^{-1}; α-aminobutyrate (27 mM) > 20%, 6×10^{-3} s^{-1} (at 37°C). This shows conclusively that these non-cognate amino acids are genuine substrates of the preparation of alanyl-tRNA synthetase. It is unlikely that the activities are due to an impurity of another aminoacyl-tRNA

in the preparation since *i*, the values of k_{cat} for glycine and alanine are too high for any impurity other than large amounts of their cognate enzymes and *ii*, the values of K_M are too high for the reactions of their cognate enzymes. Similarly, the enzyme most likely to activate α-aminobutyrate, the valyl-tRNA synthetase, exhibits a K_M value low enough to have been measured in this study (1).

Attempts to Charge $tRNA^{Ala}$ with Glycine or Serine. All attempts to detect formation of Gly-$tRNA^{Ala}$ and Ser-$tRNA^{Ala}$ using catalytic amounts of alanyl-tRNA synthetase and ^{14}C-labelled amino acids failed under steady-state conditions. Similarly, experiments with unlabelled samples of serine at high concentration using the method of Old and Jones (that is, by measuring the depression of formation of cognately-charged tRNA in an aminoacylation mixture on the addition of a non-cognate amino acid (11) showed low levels of mischarging. For example, as in the "isotopic dilution" studies discussed above, addition of serine to an aminoacylation mixture containing 30 μM ^{14}C-alanine, ATP, alanyl-tRNA synthetase and inorganic pyrophosphatase in the standard pH 7.8 buffer resulted in a 50% inhibition of charging at 430 mM serine. The concentration of serine required is so high compared with that predicted from its relative specificity in the pyrophosphate-exchange reaction that there must be an editing mechanism to remove the products of misactivation. The rates of activation of α-aminobutyrate and cysteine are so low at accessible substrate concentrations compared with the rates of hydrolysis of aminoacyl-tRNA that it was not feasible to check for mischarging with these amino acids.

Detection of Editing by the ATP-Pyrophosphatase Activity. The diagnostic test for the occurrence of an editing mechanism in the selection of amino acids by the aminoacyl-tRNA synthetases is the presence of an ATP-pyrophosphatase activity stimulated by the non-cognate amino acid as it is continually activated and the products of misactivation hydrolytically destroyed (12). It is seen in Table 2 that there is a significant ATP-pyrophosphatase activity in the presence of glycine and serine. The turnover numbers in the presence of tRNA are comparable with that for the aminoacylation reaction with alanine under similar conditions (0.8 s^{-1}). Combined with the previous results, it is apparent that there is efficient editing in the selection of glycine and serine.

Interestingly, the editing reaction occurs at a significant rate in the absence of tRNA, there being only a four to five fold stimulation by tRNA of the values of k_{cat}. Periodate-oxidized tRNA that has no acceptor

Table 2: ATP-Pyrophosphatase Activities of Alanyl-tRNA Synthetase from *E. coli*[a].

Amino acid	-tRNA		+tRNA	
	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)
Glycine	0.1 (∿0.4)[b]	0.8 (∿100)[b]	0.46	11.2
Serine	0.20	6.6	0.92	24.9
Alanine[c]	1.5×10^{-3}	-	0.04	-

[a] ATP-Pyrophosphatase reaction at 25 °C, standard pH 7.78 buffer, 2 mM [γ-^{32}P]ATP, 1.7 μM $tRNA^{Ala}$. [b] Biphasic kinetics observed with the quoted approximate values (bracketed). [c] 3 mM.

ability does not stimulate hydrolysis. The stimulation by glycine in the absence of tRNA is complicated by biphasic kinetics, there being a second K_M of about 100 mM. The kinetics in the presence of tRNA are strictly Michaelian in the substrate concentration range of 0.2-120 mM as are the kinetics of the serine-stimulated ATP-pyrophosphatase activity in both the presence and absence of tRNA. As recently discussed at length (13), there could be editing by both hydrolysis of the mischarged tRNA and the non-cognate aminoacyl adenylate.

DISCUSSION

This study demonstrates beyond reasonable doubt that the alanyl-tRNA synthetase from *E. coli* activates serine: the serine-stimulated ATP-pyrophosphate exchange activity is not caused by residual traces of alanine; serine is transferred to hydroxylamine, a commonly used acceptor nucleophile for activated esters or anhydrides. There is no *net* transfer of the serine to $tRNA^{Ala}$, however, as there is an active editing mechanism which removes the products of activation. Editing appears to occur by both hydrolysis of the non-cognate aminoacyl adenylate complex and the mischarged tRNA. In contrast, α-aminobutyrate and cysteine, which are nominally isosteric with serine, are activated far more slowly, the values of k_{cat}/K_M being some thirty times lower than that for the activation of serine (Table 1). Glycine

is also activated and subjected to editing in a manner similar to serine.

The observations on the activation of glycine are entirely as expected from extrapolation from earlier studies (7-10,12): glycine, being smaller than alanine by one methylene group, was predicted to be activated by the alanyl-tRNA synthetase with a value of k_{cat}/K_M some 200 times lower than that for the activation of alanine. The low rate of activation of α-aminobutyrate is also similar to that calculated from the relative rates of activation of isoleucine and valine by the valyl-tRNA synthetase (1). The rate constants for the activation of serine were not so readily predictable, however, since the binding of serine requires the cramming of its hydroxyl group into the body of the protein, giving rise to unknown steric effects. The better binding of serine than α-aminobutyrate is, at first sight, somewhat surprising.

Before considering the reasons for the better binding of serine in detail, it is worthwhile reviewing the binding of hydroxyl and methyl groups to the active site of an enzyme which is sufficiently large to bind all the substrates concerned without steric distortion. The analogous situation is the binding of valine, α-aminobutyrate and threonine to the valyl-tRNA synthetase (1). This enzyme presumably has a hydrophobic binding site for the amino acid side chain as does the alanyl-tRNA synthetase since both enzymes bind their cognate substrates so tightly compared with small homologues. Comparison of threonine and valine shows that the hydrophilic hydroxyl group binds far less tightly (by 3.5 kcal/mol) than the methyl group. Threonine and α-aminobutyrate bind equally well. This shows that the hydroxyl group contributes no net binding energy (relative to the hydrogen atom), the dispersion energy interactions between the hydroxyl group and the protein combined with the entropic advantage of releasing the hydrogen-bonded solvent molecules are just sufficient to compensate for the loss of the hydrogen bonds with the solvent. Thus, for a hydrophobic cavity constructed to fit the side chain of α-aminobutyrate, the expected preference of binding is expected to be α-aminobutyrate >> alanine≈serine. The results of this study on the alanyl-tRNA synthetase show that it is easier to cram a hydroxyl group into too small a cavity in an enzyme than it is to cram a methyl group. It could, perhaps, be argued that the distortion of the binding site on accommodating the overlarge substrates releases a hydrogen bonding site in the protein that is utilised by the serine hydroxyl. The hydroxyl group could, for example, be interposed between a carboxyl oxygen and an amido NH group. However, this idea runs counter to the likelihood of a binding pocket lined

with hydrophobic groups. A more reasonable explanation comes from a consideration of the steric bulk of the groups concerned. The -OH group of serine occupies a significantly smaller volume than does the CH_3- of α-aminobutyrate. First, the covalent bond distance involved is shorter (C-O bond length of serine = 1.43 Å (14), C_3-C_4 in α-aminobutyrate = 1.53 Å (15)). Second, the van der Waals' radius of the oxygen atom is 1.5 Å, whilst that of tetrahedral carbon (including the attached H) is 1.85-2.1 Å (16,17). Further, the minimum contact radius in a protein, i.e. for the closest normal distance of approach, is 1.3 Å for the oxygen atom and 1.5 Å for the tetrahedral carbon (18). These small differences are extremely important since repulsive forces between atoms follow an inverse twelfth-power law. Further evidence for the importance of small changes in bond lengths when binding to a too small active site comes from a comparison of the values of k_{cat}/K_M for the activation by the valyl-tRNA synthetase from yeast of valine with isoleucine and its nominal isostere O-methylthreonine. According to Igloi *et al* (3), the ratio of the rate constants for O-methylthreonine and valine is 3×10^{-3} whilst, according to ref (1), the ratio for isoleucine and valine is fifty times lower at 6×10^{-5} (close to the value for α-aminobutyrate and alanine in Table 1). The phenolic C-O bond in tyrosine is shorter still (bond length = 1.37 Å (19,20)) because of delocalisation, accounting for the relatively efficient activation of tyrosine by the phenylalanyl-tRNA synthetase (3).

The rate constants for cysteine, norvaline and norleucine listed in Table 1 were not obtained from measurements on "scavenged" samples of amino acids and should just be considered as upper limits that indicate rates of activation so low as to be negligible when considering their effects on accuracy.

Modifications of the Double-Sieve Model. The essence of the proposed mechanism of editing is that the relevant aminoacyl-tRNA synthetases have two active sites, one for synthesis and one for hydrolysis, and that the discrimination between cognate and non-cognate substrates depends on the relative rates of synthesis and hydrolysis. The enzyme attains its high specificity by using the differences in the structure of the competing substrates *twice*: once by discriminating against the undesired substrate in the synthetic step, and once by discriminating against the desired substrate in the destructive step (21). The double-sieve model attempts to account for the mechanism of selection at each step by reducing it to simple rules. However, as each aminoacyl-tRNA synthetase faces different problems of selection and the

enzymes do not appear to be part of one simple family, the enzymes and rules have to be divided into different groups. The first simplification is that non-cognate substrates that are larger than the cognate by just one methylene group are activated so weakly that there is no need for removal of their reaction products by editing (1). The results of the present study bear out this hypothesis. However, it must be modified for the two cases where there are natural non-cognate amino acids that are larger than the cognate by one oxygen atom, i.e., for the rejection of serine and tyrosine by the alanyl- and phenylalanyl-tRNA synthetases respectively, since the oxygen atom is not sufficiently large for it to be excluded by unfavourable steric interactions with high efficiency. The second simplification is the corollary of the first that only the products of misactivation of smaller or isosteric non-cognate substrates require editing. Again, this breaks down for the latter two enzymes. The mechanism of selection for editing presumably depends to a large extent on the relative affinities of the amino acid moiety of the products for the synthetic and hydrolytic sites. The non-cognate amino acids have a known reduced affinity for the synthetic active site. It is proposed that the cognate amino acid has a reduced affinity for the hydrolytic active site for reasons either of steric exclusion (e.g. isoleucine for a hydrolytic site tailored for valine on the isoleucyl-tRNA synthetase) or for specific chemical characteristics (e.g. valine for a hydrolytic site on the valyl-tRNA synthetase tailored for the -OH group of threonine) (1). In the examples of the alanyl- and phenylalanyl-tRNA synthetases, the synthetic and hydrolytic sites are presumably constructed that the aminoacyl side chains of the (readily) activated non-cognate substrates favour binding to the hydrolytic site. Another class of exceptions, examples of where efficient editing can be achieved by just one active site, have been recently discussed elsewhere in this journal (13). Notwithstanding the number of exceptions, the double-sieve mechanism still usefully summarises the principles of selection in editing.

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