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Evidence for the Double-Sieve Editing Mechanism in Protein Synthesis. Steric Exclusion of Isoleucine by Valyl-tRNA Synthetases†

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ABSTRACT: Evidence is presented for a simple stereochemical model, the "double sieve", by which the exquisite fidelity of the genetic coding process is preserved at the level of charging tRNA with the correct amino acid. The high accuracy in the recognition of amino acids is achieved by the synthetic and hydrolytic (editing) sites on an aminoacyl-tRNA synthetase functioning as a pair of sieves, crudely sorting the amino acids according to size as well as chemical nature. The synthetic site rejects, at a tolerable level, amino acids larger than the specific substrate; the hydrolytic site destroys the reaction products of the amino acids which are smaller than (or isosteric with) the specific substrate. Testing hypothetical editing mechanisms by mapping out the range of misactivations catalyzed by the aminoacyl-tRNA synthetases is hampered by trace impurities of the specific amino acid in preparations of the nonspecific amino acids. An enzymic method is given for scavenging these impurities. It is found that at least 97% of the apparent isoleucine-dependent pyrophosphate exchange activity of three representative valyl-tRNA synthetases is attributable to residual traces of valine. The selectivity of the enzyme from *Escherichia coli* against isoleucine is greater than 6×10^4. Combined with the known ratio of concentrations of isoleucine and valine in vivo, an error rate of less than 3×10^{-6} is calculated for the mistaken activation of isoleucine for valine. Isoleucine is thus sieved out at the activation step and the rate of formation of Ile-tRNA^{Val} is so low as not to require subsequent editing.

The selection of amino acids during protein biosynthesis is very precise. The overall error rate for the misincorporation of valine for isoleucine, which is possibly the most difficult example, is only 1/3000 (Loftfield, 1963; Loftfield & Vanderjagt, 1972). In order to maintain this accuracy at the level of aminoacylation of tRNA, certain aminoacyl-tRNA synthetases have evolved "editing" or "proofreading" mechanisms whereby the products of misactivated amino acids are removed somehow by hydrolysis (Norris & Berg, 1964; Baldwin & Berg, 1966). It has now been shown that the editing of misactivated threonine and α-aminobutyrate by valyl-tRNA synthetases occurs after the tRNA^{Val} has been misacylated (Fersht & Kaethner, 1976; Fersht & Dingwall, 1979a): the amino acid is activated by the enzyme (eq 1) and is transferred to the tRNA (eq 2) in the normal way but the misacylated tRNA is rapidly deacylated by a separate hydrolytic active site on the enzyme before the tRNA is released into solution (eq 3).

$$\text{E·Thr·ATP} \rightarrow \text{E·Thr–AMP} + \text{PP}_i \quad (1)$$

$$\text{E·Thr–AMP} + \text{tRNA}^{Val} \rightarrow \text{E·Thr–tRNA}^{Val} + \text{AMP} \quad (2)$$

$$\text{E·Thr–tRNA}^{Val} \rightarrow \text{E} + \text{Thr} + \text{tRNA}^{Val} \quad (3)$$

Our working hypothesis for predicting when misactivations will occur and where editing mechanisms are necessary is based on a combination of ideas of steric exclusion and goodness of fit (Fersht, 1977a). It is reasoned that amino acids larger than the specific substrate of an aminoacyl-tRNA synthetase will be excluded, at a tolerable level, from binding productively to the active site by steric hindrance. On the other hand, smaller amino acids must be able to bind productively and react but the reaction rates will be lower in proportion to the decrease in binding energy. It is suggested that editing is required only for rejection of the smaller amino acids—the "double-sieve" hypothesis (Fersht, 1977a; Fersht & Dingwall, 1978).

Whereas there is a simple theory to account for the binding of smaller substrates to a larger active site (Fersht 1974, 1977a), there is little quantitative knowledge of the steric hindrance to the binding of larger substrates. It is clear that phenylalanine, for example, will be completely excluded from the active site of the valyl-tRNA synthetase, but the degree of exclusion of isoleucine is unknown. It has been reported that the valyl-tRNA synthetase from *Escherichia coli* catalyzes the pyrophosphate exchange reaction in the presence of isoleucine with values of V_{max} as high as 10–22% of that with valine (Loftfield & Eigner, 1966; Yaniv & Gros, 1969). The enzyme from yeast has recently been reported to catalyze the isoleucine-dependent reaction with V_{max} = 57% of that for valine and K_M = 7.1 mM (compared with 0.17 mM for valine) (Igloi et al., 1978). If the latter is so, then an efficient editing mechanism is required to correct the mistaken activation of

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0006-2960/79/0418-2627$01.00/0

isoleucine, and the simple double-sieve scheme breaks down.

In order to determine whether the rate of activation of a competing amino acid by an aminoacyl-tRNA synthetase (eq 1) is sufficiently high to warrant an editing mechanism, it is clearly important to use amino acid preparations of extreme purity since error rates as low as about $1/10^5$ must be detected. This problem is particularly severe when measuring the activation reaction by the conventional pyrophosphate exchange method since the amino acid is not consumed and so trace impurities will recycle. In our experience, commercial samples of amino acids often contain significant trace quantities of contaminants even after recrystallization. We have shown, for example, that isoleucine contains residual amounts of methionine which can be removed by treatment with Raney nickel (Fersht & Dingwall, 1978, 1979c). In the present paper, valine is removed from commercial samples of isoleucine by an enzymic method. Advantage is taken of the ability of the aminoacyl-tRNA synthetases to transfer amino acids to hydroxylamine (eq 4) to scavenge traces of activatable amino

$$E + RCH(NH_2)CO_2H + ATP + NH_2OH \rightarrow E + RCH(NH_2)CONHOH + AMP + PP_i \quad (4)$$

acids. The contaminating amino acid, in this case valine, is removed as the hydroxamate by column chromatography. In this way, the upper limit for the activation of isoleucine by various valyl-tRNA synthetases can be accurately determined by pyrophosphate exchange and thereby used to judge the requirement for an editing mechanism. We summarize the evidence for the double-sieve hypothesis.

Materials and Methods

Materials. The preparations of valyl-tRNA synthetases and $tRNA^{Val}$ were those previously used in this laboratory and are described elsewhere (Fersht & Kaethner, 1976; Fersht & Dingwall, 1979a; Fersht & Mulvey, 1977a,b). All enzymes were homogeneous according to accepted criteria and greater than 90% active according to active site titration (Fersht et al., 1975). The valyl acceptance of the $tRNA^{Val}$ from *Escherichia coli* was 480, and that from *Bacillus stearothermophilus* was 1400 pmol/A_{260}. Amino acids were obtained from Sigma or BDH and recrystallized from ethanol/water before use. [^{14}C]Val (280 Ci/mol) and [^{14}C]Ile (348 Ci/mol) were obtained from the Radiochemical Centre, Amersham, U.K.

Treatment of Isoleucine with Hydroxylamine. A solution (5 mL) containing ATP (10 mM), $MgCl_2$ (10 mM), mercaptoethanol (14 mM), phenylmethanesulfonyl fluoride (0.1 mM), inorganic pyrophosphatase (1 unit/mL), H_2NOH·HCl (1 M), NaOH (0.8 M), isoleucine (114 mM), and valyl-tRNA synthetase (*B. stearothermophilus*, 1 μM) was incubated for either 8, 16, or 32 h at 37 °C. One sample was incubated for 72 h with fresh additions of ATP, enzymes, and hydroxylamine after 24 and 48 h. The reaction was quenched by the addition of acetic acid to 4 M and the resultant solution (50 mL) loaded onto a column (4 × 15 cm) of SP-Sephadex C-25 equilibrated with 4 M acetic acid. The isoleucine was eluted with a gradient of acetic acid (4 M)–pyridine (0.04 M) and acetic acid (1 M)–pyridine (0.5 M), and after lyophylization, assayed by ninhydrin.

Kinetic Procedures. Pyrophosphate exchange, aminoacylation, and ATP/pyrophosphatase activities were measured at 25 °C and pH 7.78 in buffers containing Tris-Cl (144 mM), $MgCl_2$ (10 mM), mercaptoethanol (14 mM), and phenylmethanesulfonyl fluoride (0.1 mM) by conventional procedures (Baldwin & Berg, 1966; Fersht & Kaethner, 1976; Fersht, 1977b).

Table I: Effect of Impurities in Isoleucine on the Pyrophosphate Exchange Activity of Valyl-tRNA Synthetases[a]

amino acid	incubation time[d] (h)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
		(a) Enzyme from *E. coli*		
Val	0	61	0.083	7.4×10^5
Ile	0	4.2	8.3	506
	8	0.58		
	16	0.44	7.9	56
	32	0.15	10	15
	72[c]	0.15	15	10
		(b) Enzyme from *B. stearothermophilus*		
Val	0	33	0.03	1.1×10^6
Ile	0	1.9		
	8	0.22[b]		
	16	0.15[b]		
	32	0.06	14	4
	72[c]	0.06	12	5
		(c) Enzyme from Yeast		
Val	0	37	0.088	4.2×10^5
Ile	0	7		
	16	1.6	28	57
	32		>100	26
	72[c]		>100	24

[a] Pyrophosphate exchange reaction at 25 °C, standard pH 7.78 buffer, 2 mM ATP, and 2 mM [^{32}P]pyrophosphate stimulated by the amino acid in column 1. [b] 27 mM isoleucine, not saturating. [c] Fresh addition of reagents after 24 and 48 h. [d] With hydroxylamine and ATP.

Results

(*a*) *The Pyrophosphate Exchange Activity in the Presence of Isoleucine Is Mainly Due to an Impurity.* Commercial samples of isoleucine from various sources stimulated pyrophosphate exchange activity with the three enzymes studied. The activity was little reduced after two successive recrystallizations of the isoleucine from ethanol/water. However, the activity was reduced 30-fold after prolonged treatment of the isoleucine with hydroxylamine and ATP (Table I). The decrease in reactivity was not caused by the carrying over of an inhibitor from the hydroxylamine treatment since the addition of valine to the treated isoleucine stimulated rapid pyrophosphate exchange. Instead, the loss of activity is caused by the removal of an impurity which is almost certainly valine (see below). There is no further decrease in activity after 32-h treatment with hydroxylamine and ATP. Whether the residual activity represents the limits of effectiveness of the method for removal of valine or is genuinely caused by an isoleucine-dependent pyrophosphate exchange activity of the valyl-tRNA synthetase is not known. The activity is certainly not caused by any isoleucyl-tRNA synthetase in the preparations of enzymes since the values of K_M, about 10 mM, are far too high. The residual activities thus represent the upper limits of any activation of isoleucine by the valyl-tRNA synthetases.

The value of K_M for isoleucine in the reaction catalyzed by the enzyme for *E. coli* remains constant at about 10 mM during the purification process (Table I). This is close to the value of 15 mM we find for K_I for the inhibition of the valine-stimulated pyrophosphate exchange by isoleucine. This suggests that isoleucine does bind to the valyl-tRNA synthetase with a dissociation constant of about 10–20 mM.

(*b*) *The Impurity Will Aminoacylate $tRNA^{Val}$ and Has the Characteristics of Valine.* Prolonged incubation of $tRNA^{Val}$ (from *E. coli* or *B. stearothermophilus*) with [^{14}C]Ile, ATP, inorganic pyrophosphatase, and the appropriate valyl-tRNA

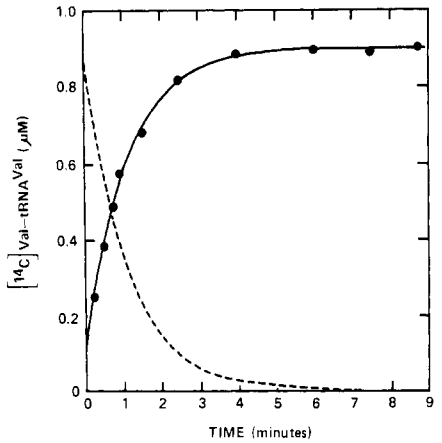

FIGURE 1: Back-titration of $tRNA^{Val}$ (*B. stearothermophilus*) which had been preincubated with isoleucine, ATP, valyl-tRNA synthetase, and inorganic pyrophosphatase (see text). (Solid curve) Aminoacylation of $tRNA^{Val}$ (0.9 μM) in the presence of valyl-tRNA synthetase (4 μM), [^{14}C]Val (45 μM), ATP (2 mM), and inorganic pyrophosphatase. Broken curve, deacylation of [^{14}C]Val-$tRNA^{Val}$ (0.9 μM) in the presence of valyl-tRNA synthetase (4 μM), valine (45 μM), ATP (2 mM), and inorganic pyrophosphatase. Both reactions at pH 7.78, 25 °C, in the presence of 10 mM $MgCl_2$ and 14 mM mercaptoethanol.

synthetase leads to no charging of the tRNA. An aminoacyl-tRNA is formed, however, on incubation with unlabeled isoleucine under the same conditions. But this is almost certainly Val-$tRNA^{Val}$, formed from contaminating valine, rather than Ile-$tRNA^{Val}$. The aminoacyl-tRNA was detected by back titration (Igloi et al., 1977). For example, 12.4 μM $tRNA^{Val}$, 0.5 μM valyl-tRNA synthetase (*B. stearothermophilus*), 50 mM isoleucine, 2 mM ATP, and inorganic pyrophosphatase (1 unit/mL) were incubated for 10 min in the pH 7.78 buffer at 25 °C, and the tRNA was isolated by phenol extraction. On addition of the tRNA to an aminoacylation mixture containing [^{14}C]Val (Figure 1), 10% was rapidly aminoacylated but the remaining 90% only slowly. The rate of aminoacylation of the 90% varied with enzyme concentration in a manner identical with that of the valyl-tRNA synthetase-catalyzed deacylation of Val-$tRNA^{Val}$. With an excess of enzyme (4 μM) over tRNA (0.9 μM), 45 μM [^{14}C]Val, 2 mM ATP, and pyrophosphatase at pH 7.78 and 25 °C, aminoacylation occurred in a first-order process with a rate constant of 0.015 s^{-1}. The deacylation rate constant of [^{14}C]Val-$tRNA^{Val}$ under the same conditions (except that 45 μM unlabelled valine was used) was also found to be 0.015 s^{-1}. Thus, 90% of the tRNA appears to have been converted to Val-$tRNA^{Val}$ and this has to be deacylated before aminoacylation with [^{14}C]Val can occur.

The amount of valine in the isoleucine may be estimated from the dilution of the specific activity of [^{14}C]Val. For example, the addition of 50 mM (twice-recrystallized) isoleucine to an aminoacylation mixture containing 18.5 μM [^{14}C]Val led to a 57% reduction in the amount of radioactivity transferred to the $tRNA^{Val}$. This corresponds to the presence of 25 μM valine in the isoleucine, only 0.05%.

(*c*) *Activation of Other Amino Acids.* The data for the pyrophosphate exchange reaction stimulated by amino acids smaller than or isosteric with valine are listed in Table II. In the light of present experience, the rate constants must be considered as upper limits of the true activities. The data for the more reactive substrates almost certainly represent the true activities since an extensive kinetic analysis of the reactions of threonine and α-aminobutyrate is consistent with these values (Fersht & Dingwall, 1979a).

Table II: Activation of Amino Acids Catalyzed by the Valyl-tRNA Synthetase from *E. coli*[a]

amino acid	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})	"discrimination" (rel value of k_{cat}/K_M)
Ile	≤ 0.15	12	≤ 12	$\leq 2 \times 10^{-5}$
Val	61	0.083	7.4×10^5	1
Thr	11	3.7	3.0×10^3	4×10^{-3}
α-aminobutyrate	25	6.5	3.8×10^3	5×10^{-3}
Ala	2.1	27	78	1×10^{-4}
Gly			7×10^{-2}	9×10^{-8}

[a] Pyrophosphate exchange reaction as for Table I.

(*d*) *ATP/Pyrophosphatase Activities.* When an amino acid is activated and hydrolytic editing occurs, as in eq 1, 2, and 3, there is an overall ATP/pyrophosphatase activity (Baldwin & Berg, 1966). The data for threonine and α-aminobutyrate are presented elsewhere (Fersht & Kaethner, 1976; Fersht & Dingwall, 1979a). In this study, we find that there is significant ATP/pyrophosphatase activity in the presence of alanine, $tRNA^{Val}$, and the valyl-tRNA synthetase (*E. coli*). At 50 mM alanine, pH 7.78, and 25 °C, the specific rate constant is 0.8 mol/(mol of enzyme)$^{-1}$ s^{-1}. Thus the misactivation of alanine is actively corrected by hydrolytic editing.

(*e*) *Concerning the Interpretation of Values of k_{cat} and K_M.* If the value of k_{cat} for the activation of a competitive amino acid is lower than that for the activation of the specific substrate, it is tempting to conclude that the activity is not caused by the presence of the specific substrate as an impurity in the competitive substrate. There is no justification for this. It may be shown, for example, that if the competitive substrate is just a competitive inhibitor which binds to the enzyme with a dissociation constant K_I, and the ratio of the specific substrate to the inhibitor is x, then

$$(k_{cat})_{obsd} = k_{cat}[x/(x + K_M/K_I)] \quad (5)$$

and

$$(K_M)_{obsd} = K_M/(x + K_M/K_I) \quad (6)$$

(where K_M and k_{cat} are the values for the pure specific substrate). Equation 6 also holds when the competitive amino acid is also a substrate. When x is very small, eq 6 reduces to $K_M = K_I$. This is presumably why the value of K_M for the activation of isoleucine by the enzyme from *Escherichia coli* remains constant throughout the purification procedure, at about the observed value of K_I, whilst the value of k_{cat} decreases. The value of k_{cat} with the untreated isoleucine (where the activity is mainly due to the presence of valine) is calculated from eq 6, the measured K_I, and the fraction of valine determined by the radioactive dilution procedure, to be 5.06 s^{-1}, very close to the observed value of 4.2 s^{-1}.

(*f*) *Is Ile-$tRNA^{Val}$ Formed?* The failure to detect any [^{14}C]Ile-$tRNA^{Val}$ in an aminoacylation mixture containing 20 μM [^{14}C]Ile does not mean that the isoleucine is not transferred to the tRNA. The rate of activation of the amino acid at that concentration, less than 3×10^{-4} mol of amino acid (mol of enzyme)$^{-1}$ s^{-1} (from data in Table I), is less than that expected for the spontaneous hydrolysis of the aminoacyl-tRNA under these conditions and so little charged material is expected to accumulate. Similarly, the lack of trapping of the charged tRNA does not mean that there is an editing mechanism which actively hydrolyzes the misacylated tRNA, but may just reflect the normal instability of the ester bond.

(*g*) *Concerning the Igloi et al. (1978) Test for Misactivation.* Igloi et al. (1978) have described a series of tests for misactivation. An amino acid is considered to be misactivated

and then rejected by "chemical proofreading" if it stimulates pyrophosphate exchange, an ATP/pyrophosphatase activity, is not apparently transferred to tRNA, but is transferred to tRNA in which the accepting hydroxyl group is replaced by an amino group (NH_2-tRNA). Applying this to the case of isoleucine and the valyl-tRNA synthetase from yeast, it was concluded that the amino acid is rapidly activated and then "chemically proofread". The reason for the breakdown of the test is that it must be applied quantitatively, measuring all the relevant rate constants, rather than qualitatively. In particular, the rate of misaminoacylation of the NH_2-tRNA must be measured and shown to be significantly high. As we have found for isoleucine and valyl-tRNA synthetases in this study, the presence of impurities of the cognate amino acid (or any other activatable amino acid) in the noncognate (for example, valine in the isoleucine) will give an overestimate of the pyrophosphate exchange reaction. An apparent ATP/pyrophosphatase activity will arise through the normal formation of the cognate-charged tRNA followed by its hydrolysis (or also by the presence of any threonine of alanine in the isoleucine). By using pure ^{14}C-labeled isoleucine, no [^{14}C]-Ile-tRNA is expected to accumulate as described in section f (although back-titration with the unlabeled isoleucine leads to an *apparent* mischarging of the $tRNA^{Val}$ (Figure 1)).

Controls must also be performed to test whether the transfer of the amino acid to NH_2-tRNA is part of the normal catalytic cycle or is the adventitious result of the dissociation of, for example, a slowly formed E·[^{14}C]Ile–AMP complex followed by the nonenzymic reaction of the aminoacyl adenylate with the amino group of the NH_2-tRNA (amino groups compete with water for activated carboxyl groups at even micromolar concentrations (Jencks & Gilchrist, 1968)).

Discussion

The activation of isoleucine by the valyl-tRNA synthetase has been found previously to proceed with values of k_{cat} varying from 3 to 57% of that for the valine-dependent reaction (Owens & Bell, 1970; Loftfield & Eigner, 1966; Yaniv & Gros, 1969; Igloi et al., 1978). It has been suggested that the activity may be caused by the presence of valine in the commercial samples of isoleucine (Owens & Bell, 1970). The results of this study show that this is so. Removal of the valine (or any other amino acid which may react) by conversion to the hydroxamic acid reduces the activity over 30-fold. Once contaminants have been removed, particularly the cognate amino acid, an upper limit on the activation can be obtained by the pyrophosphate exchange technique to assess the need for an editing mechanism. The method for removal of reactive impurities should be quite general and easier for most other cases since the valyl-tRNA synthetase is particularly unreactive in transferring its amino acid to hydroxylamine (Hirsch & Lipmann, 1968).

Calculation of Misincorporation Frequencies. The relevant kinetic quantity for calculating error rates is k_{cat}/K_M since the relative reaction rate of two substrates A and B competing for the same enzyme is given by (Fersht, 1974, 1977a)

$$v_A/v_B = (k_{cat}/K_M)_A[A]/(k_{cat}/K_M)_B[B] \qquad (7)$$

The relative values of k_{cat}/K_M for the activation of valine and isoleucine catalyzed by the valyl-tRNA synthetases from *E. coli*, *B. stearothermophilus*, and yeast are greater than 6×10^4, 2.4×10^5, and 1.8×10^4, respectively. By using eq 7 and the known ratio of valine to isoleucine in *E. coli* (Raunio & Rosenqvist, 1970), it is calculated that, if all the isoleucine activated by the valyl-tRNA synthetase is incorporated into proteins, the *upper limit* of the error rate for the misincorporation of isoleucine for valine would be 3×10^{-6}. This is two orders of magnitude less than that found for the misincorporations of valine for isoleucine that survive to be measured in rabbit globin (Loftfield & Vanderjagt, 1972). Isoleucine may thus be rejected at the activation step by the valyl-tRNA synthetase without the need for an editing mechanism to hydrolyze the mischarged tRNA.

The Double-Sieve Sorting Mechanism. The selection of amino acids by the aminoacyl-tRNA synthetases has been likened to the action of sorting by a pair of sieves (Fersht, 1977a). The amino acids are crudely sorted by size. It is proposed that amino acids larger than the specific substrate are adequately rejected by the activation site in a single step by steric hindrance but isosteric and smaller substrates are accepted. The principle is used a second time for selection for hydrolytic editing. The hydrolytic site accepts *only* (or strongly prefers) substrates smaller than or, in certain cases, isosteric with the specific substrate, leaving only the desired product intact.

Superimposed upon the selection by size is a discrimination amongst the isosteric and smaller substrates on the grounds of stereochemical fit and specific binding interactions. The incorrect substrates bind less well to the enzyme and so there are lower values of k_{cat}/K_M for activation (Fersht, 1974, 1977a).

On this model, the amino acid competitors of a particular substrate may be divided into three cases, depending on size. Only smaller or isosteric substrates require the editing function. The three cases are as follows.

(*a*) *Amino Acids Larger than the Specific Substrate.* The results for this study show that the aminoacyl-tRNA synthetases can discriminate adequately against natural substrates which are just one methylene group larger than the specific substrate without recourse to an editing mechanism for deacylating the mischarged tRNA. However, when challenged with unnatural substrates, lower specificity may be found. For example, the methionyl-tRNA synthetase activities ethionine (Trupin et al., 1962) and the valyl-tRNA synthetase from yeast is reported to activate *O*-methylthreonine (Igloi et al., 1977).

(*b*) *Amino Acids Smaller than the Specific Substrate.* As seen in Table II, smaller amino acids are activated but the value of k_{cat}/K_M falls off rapidly with decreasing size of side chain as predicted by theory (Fersht, 1974, 1977a). But, as one methylene group contributes up to only a factor of 200–300 toward k_{cat}/K_M, editing mechanisms are required to remove activation products of amino acids which are one methylene group shorter than the specific substrate. The editing process extends to even smaller homologues; we find in this study that alanine is actively edited by the valyl-tRNA synthetase. The methionyl-tRNA synthetase efficiently edits in the presence of homocysteine and norvaline (Fersht & Dingwall, 1979c). Other enzymes which clearly require editing of smaller substrates are: alanyl-tRNA synthetase (against glycine), threonyl-tRNA synthetase (against serine), etc.

Glycine, because of its small size, must be activated by all the aminoacyl-tRNA synthetases. But, by the same token, the rate is too small in most cases to be of importance. Similarly, we find that the specificity of the cysteinyl-tRNA synthetase for cysteine (relative to serine, alanine, and α-aminobutyrate) in the activation step is so high that no editing is required (Fersht & Dingwall, 1979b).

(*c*) *Amino Acids Isosteric with the Specific Substrate.* Size alone is inadequate for sorting isosteres and so the enzyme must invoke the specific binding characteristics of the substrates. For this reason, the most common unnatural amino

acids that are incorporated into proteins are those which are isosteric with a naturally occurring amino acid (e.g., canavine for arginine, 7-azatryptophan for tryptophan, β-chloro-α-aminobutyrate for valine). The unnatural isosteres pass the test of both sieves. In the case of natural isosteres, such as threonine with valine, the valyl-tRNA synthetase first discriminates against threonine by forcing its –OH group to bind in a hydrophobic region of the activation site and then, somehow, uses the –OH group to draw threonine into the hydrolytic site. Our proposal is that the hydrolytic site is partly blocked by a bound water molecule which inhibits the binding of valine. There is room for smaller amino acids to be sieved in the normal way and threonine can also bind by using its –OH group to displace the bound water (Fersht & Dingwall, 1979a). (For editing of misactivated valine by the threonyl-tRNA synthetase, there is presumably a hydrophobic editing site which strongly prefers the valyl residue.)

Steric Requirements at the Hydrolytic Site. Further evidence for the double-sieve mechanism comes from studies on the aminoacyl-tRNA synthetase catalyzed deacylation of aminoacyl-tRNA. The editing reaction of the valyl-tRNA synthetase occurs by the hydrolysis of the mischarged tRNA and some, if not all, of the editing by the isoleucyl-tRNA synthetase takes place at this stage also (Fersht, 1977b). The only aminoacyl-tRNAs that are rapidly deacylated by the aminoacyl-tRNA synthetase cognate to the tRNA are those which are aminoacylated with smaller or isosteric amino acids (for example: Val-tRNA^{Phe}, k_{cat} = 4 s^{-1} (Bonnet & Ebel, 1974); Ile-tRNA^{Phe}, 2 s^{-1} (Yarus, 1972); Val-tRNA^{Ile}, 10 s^{-1} (Fersht, 1977b); Thr-tRNA^{Val}, 40 s^{-1} (Fersht & Kaethner, 1976); α-aminobutyryl-tRNA^{Val}, 50 s^{-1} (Fersht & Dingwall, 1979a)). All other aminoacyl-tRNAs are deacylated only slowly in the presence of the parent enzymes (Bonnet & Ebel, 1974; Bonnet, 1974; Sourgoutchoff et al., 1974; Schreier & Schimmel, 1972).

In summary, there is strong evidence to support the double-sieve model as the means by which exquisite discrimination is achieved during the esterification of a specific tRNA despite a wide range of competing similar substrates. Amino acids larger than the true substrate are screened out at the activation step by steric hindrance. This has been made clear by enzymically removing contaminating cognate amino acids from commercial samples of "pure" amino acid. Amino acids which are smaller than the true substrate can pass the first screen and are activated and mischarged at often significant rates; however, they are edited, or proofread, by a second, hydrolytic, center which sterically impedes access of the cognate aminoacyl-tRNA. Naturally occurring isosteric amino acids which cannot be sieved on size alone are activated more slowly and the mischarged tRNA deacylated much faster. The net result is an acceptable level of cognate specificity. This simple two-step process is the basis by which the fidelity of the genetic code is preserved during aminoacylation of tRNA despite the seemingly small chemical distinction between competing amino acid substrates.

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