An Editing Mechanism for the Methionyl-tRNA Synthetase in the Selection of Amino Acids in Protein Synthesis†

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ABSTRACT: Trace impurities of methionine in commercial samples of amino acids have obscured the pattern of misactivations catalyzed by methionyl-tRNA synthetases and have concealed the existence of an editing mechanism. Recrystallized samples of α-aminobutyrate, valine, and isoleucine, for example, are found to stimulate the exchange of pyrophosphate into ATP catalyzed by the enzyme from *Bacillus stearothermophilus* with values of k_{cat} approaching that for the methionine-stimulated reaction. Following removal of methionine from the nonspecific amino acids by treatment with Raney nickel, activity is abolished with the branched side chain amino acids and considerably reduced with certain unbranched side chain amino acids. There is an editing mechanism for the removal of the misactivation of amino acids with smaller side chains than methionine: the enzymes from *Escherichia coli* and *B. stearothermophilus* do not catalyze the transfer of homocysteine or norvaline to $tRNA^{Met}$ but instead catalyze an ATP–pyrophosphatase activity. The turnover number for the ATP–pyrophosphatase activity in the presence of homocysteine and $tRNA^{Met}$ is similar to that for the aminoacylation of the tRNA with methionine. The amino acids are sorted by size in accord with the "double-sieve" editing mechanism. Amino acids sterically smaller than methionine are activated with reduced values of k_{cat}/K_M and their products are removed by hydrolytic editing. The sterically larger ethionine is activated, and its products are resistant to hydrolysis. Norleucine, which although nominally isosteric with methionine is slightly smaller, is on the borderline of being edited: there is both transfer to $tRNA^{Met}$ and an amino acid stimulated ATP/pyrophosphatase activity. The nonaccepting terminal hydroxyl group of $tRNA^{Met}$ is not essential for the editing reaction. The synthetic $tRNA^{Met}$ which is deoxy in this position does not accept homocysteine or norvaline but stimulates the amino acid dependent ATP–pyrophosphatase reaction.

How widespread are editing mechanisms for the rejection of misactivated amino acids by the aminoacyl-tRNA synthetases? The isoleucyl- and valyl-tRNA synthetases actively edit (Baldwin & Berg, 1966; Fersht & Kaethner, 1976; Igloi et al., 1977); the cysteinyl-tRNA synthetase appears not to do so (Fersht & Dingwall, 1979b). Our working hypothesis is that editing mechanisms are required whenever there are smaller or natural isosteric amino acids that can bind effectively to the active site of the enzyme (Fersht, 1977a). By this token, it would be expected that the methionyl-tRNA synthetase should have an editing mechanism since it is known to catalyze the pyrophosphate exchange reaction at an appreciable rate in the presence of amino acids with unbranched side chains (e.g., Old & Jones, 1977). In particular, homocysteine, the precursor of methionine in its biosynthesis, differs only by being shorter by one methylene group. It is well documented that the larger ethionine and the unnatural isostere norleucine are transferred to $tRNA^{Met}$ (Trupin et al., 1966) and it has been reported that homocysteine, norvaline, and α-aminobutyrate are also successfully transferred (Old & Jones, 1976, 1977).

It is our experience that commercial samples of amino acids sometimes contain trace impurities of other amino acids which are resistant to removal. These may give rise to artifactual results when mapping out the nature of activations catalyzed by aminoacyl-tRNA synthetases and also obscure the requirements for editing mechanisms. For example, we have recently found that 97% of the apparent isoleucine-dependent pyrophosphate exchange activity of the valyl-tRNA synthetase is attributable to residual traces of valine (Fersht & Dingwall, unpublished experiments). In the present paper, we remove impurities of methionine from various amino acids by treatment with Raney nickel. It is shown that presence of an editing mechanism is hidden by these impurities.

Experimental Section

Materials. The methionyl-tRNA synthetase from *Bacillus stearothermophilus* was obtained as described previously (Mulvey & Fersht, 1976). The methionyl-tRNA synthetase from *E. coli* and its proteolytic fragment (Cassio & Waller, 1971) were generous gifts from Dr. C. J. Bruton. All enzymes were purified to homogeneity. Each ran as a single band on polyacrylamide gel electrophoresis with sodium dodecyl sulfate. The concentrations of the enzyme solutions were calculated from the molar absorbances and molecular weights using the previously reported values (Fayat et al., 1974; Mulvey & Fersht, 1976). Active site titration by nitrocellulose disk filtration at pH 7.78 was performed as previously described for the native enzymes (Mulvey & Fersht, 1976) but proved unsatisfactory for the fragment from *E. coli*, as did the "burst" method of Fersht et al. (1975) because of the rapid dissociation of the E·Met-AMP complex. $tRNA^{Met}$ from *E. coli* (methionine acceptance = 800 pmol/A_{260}) was obtained from the Microbiological Research Establishment, Porton Down, Wiltshire, England. $tRNA^{Met}_F$ from *B. stearothermophilus* (methionine acceptance = 1200 pmol/A_{260}) and $tRNA^{Met}_M$ (1580 pmol/A_{260}) are described elsewhere (Mulvey & Fersht, 1978). Amino acids were obtained from Sigma and recrystallized from ethanol–water prior to use. ^{14}C- and ^{35}S-labeled methionine and ^{35}S-labeled homocystine were obtained from the Radiochemical Centre, Amersham, England. ^{14}C-labeled ethionine was obtained from C.I.S., Gif-sur-Yvette, France. ^{3}H-Labeled L-α-aminobutyrate and L-norleucine were prepared from the unlabeled materials by the TR1 labeling service of the Radiochemical Centre. The crude material was purified by chromatography on SP Sephadex C-25 as described previously (Fersht & Dingwall, 1979a). The resultant

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[^{3}H]Nle[1] was 92.5% radiochemically pure with a specific activity of 120 Ci/mol. The α-[^{3}H]But was 97% radiochemically pure with a specific activity of 248 Ci/mol. Comparison of the optical rotation of the products with the starting materials revealed that no racemization had occurred during the preparation. [^{14}C]Norvaline was synthesized as described for α-[^{14}C]But (Fersht & Dingwall, 1979a).

3′-Deoxy-tRNA^{Met} from E. coli was prepared according to a combination of known procedures. The tRNA^{Met} (800 pmol/A_{260} of methionine acceptance) was subjected to periodate oxidation, addition of glycerol to destroy excess periodate, removal of terminal sugar ring by incubation in lysine-Cl buffer, removal of terminal phosphate by alkaline phosphatase (Tal et al., 1972), addition of 3′-deoxy-AMP by incubation with cordycepin triphosphate (Sigma) and tRNA:nucleotidyl transferase (kind gift of Dr. John Smith), repetition of periodate oxidation and lysine incubation treatment to remove any terminal adenosine incorporated from any ATP present in the cordycepin triphosphate (Sprinzl & Cramer, 1975). The resultant material had a methionine acceptance of 550 pmol/A_{260}.

Treatment of Amino Acids with Raney Nickel. Methionine in commercial amino acid preparations was converted to α-aminobutyrate according to Fonken & Mozingo (1947). An aqueous solution of the amino acid was heated at 90–95 °C for 40 min with Raney nickel (1 g/g of amino acid). After cooling and filtering off the Raney nickel, the amino acid was precipitated by the addition of ethanol. The residual nickel was removed by redissolving the acids in water and repeatedly extracting with a 0.1% of dithizone in CCl_4 until the organic layer remained green. The integrity of the amino acid was checked by amino acid analysis.

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

Pyrophosphate exchange, aminoacylation, and ATP–pyrophosphatase activities were measured as described by Fersht & Kaethner (1976) and Fersht (1977b). The enzyme-catalyzed deacylation of aminoacyl-tRNA was measured under single-turnover conditions of [E] >> [aa-tRNA] (Fersht & Dingwall, 1979a). Radioactively labeled aminoacyl-tRNA was assayed by precipitation with trichloroacetic acid onto nitrocellulose (Schleicher & Schull BA 85) or glass fiber (Whatman GF/C) filters, washing with trichloroacetic acid containing unlabeled amino acids, and, after drying, measuring the radioactivity by scintillation counting using a toluene-based scintillant.

Unless otherwise stated, tRNA$^{Met}_F$ from *B. stearothermophilus* was used for the kinetic studies.

Elongation factor Tu from *E. coli* was converted to the Tu–GTP complex as described previously (Mulvey & Fersht, 1977). The concentration of Tu–GTP was measured either as previously described by the protection of Ile-tRNA against the isoleucyl-tRNA synthetase catalyzed hydrolysis or, more conveniently, by the protection of Val-tRNA^{Val}. To a solution of Tu–GTP complex (~0.5 μM) in the standard pH 7.78 buffer containing GTP (200 μM), phosphoenolpyruvate (5 mM), and pyruvate kinase (2 units/mL) was added excess [^{14}C]Val-tRNA^{Val} (~1 μM), followed by valyl-tRNA synthetase (~0.3 μM). Samples were periodically taken, quenched, and assayed for [^{14}C]Val-tRNA^{Val}. After a sufficient period had elapsed for all of the free [^{14}C]Val-tRNA^{Val} to be hydrolyzed (~20 min), the time course was extrapolated back to zero time to give the concentration of Tu–GTP that will bind aminoacyl-tRNA.

Attempt to Detect Transient Formation of [^{35}S]Homocysteinyl-tRNA^{Met} (E. coli). One syringe of the quenched flow apparatus (Fersht & Jakes, 1975) contained tRNA^{Met} (12.85 μM) in the standard pH 7.78 buffer; the other syringe contained [^{35}S]homocysteine (62 μM), dithiothreitol (10 mM) ATP (4 mM), methionyl-tRNA synthetase (1.1 μM), and inorganic pyrophosphatase (1 unit/mL) in the same buffer. Samples were periodically mixed and quenched, and the precipitated tRNA was assayed for radioactive incorporation.

Attempted Formation and Isolation of α-[^{3}H]But-tRNA^{Met}. A solution (0.2 mL) of tRNA^{Met} (5 μM), methionyl-tRNA synthetase (85 nM), α-[^{3}H]But (45 mM, 248 Ci/mol), ATP (2 mM), and inorganic pyrophosphatase (0.5 unit) in the standard pH 7.78 buffer was incubated at 37 °C. After 10 or 20 min, the solution was cooled to 0 °C and the pH lowered by the addition of sodium acetate buffer (0.04 mL, pH 5, 2 M). The solution was phenol extracted and the tRNA precipiated from the aqueous layer by the addition of 2 volumes of ethanol, followed by cooling to −20 °C. The precipitate was washed twice with 75% ethanol and then desalted on Sephadex G-25 equilibrated with 10 mM $MgCl_2$ (pH ~5). The procedure was checked by substituting [^{35}S]methionine (50 μM) for the α-aminobutyrate.

Similar experiments were performed with 3′-deoxy-tRNA^{Met} (*E. coli*) and the tRNA and enzyme from *B. stearothermophilus*. The procedure was repeated using [^{14}C]norvaline (3 mM), incubating at 25 °C for 30 min (*E. coli* system).

The putative α-[^{3}H]But-tRNA^{Met} was deacylated for analytical purposes by incubating 200 μL of solution containing 2.1 A_{260} units of aminoacyl-tRNA, 10 mM $MgCl_2$, and 56 mM pyridine at pH 7.8 for 4 h at 25 °C. The bulk of the tRNA was removed by precipitation with 2 volumes of ethanol. After freeze-drying the supernatant, the amino acid was analyzed on a Durrum amino acid analyzer converted for radioactive use (Walker et al., 1978).

Depression of the Formation of [^{35}S]Met-tRNA^{Met} by Amino Acids and Analogues. A solution (0.5 mL) containing tRNA^{Met} (0.51 μM) and methionyl-tRNA synthetase (36 nM) from *E. coli* [^{35}S]Met (2.78 μM, 380 Ci/mol), inorganic pyrophosphatase (0.25 unit), and ATP (2 mM) in the standard pH 7.78 buffer was incubated at 37 °C. Samples (50 μL) were periodically quenched with trichloroacetic acid, and the precipitated charged tRNA was collected and assayed by scintillation counting. The procedure was repeated in the presence of added DL-methioninol or α-aminobutyrate or homocysteine. Some experiments were repeated using 25 mM ATP or 25 mM ATP and 25 mM $MgCl_2$.

Results

a. Pyrophosphate Exchange Activity. The methionyl-tRNA synthetase from *B. stearothermophilus* was found to catalyze the pyrophosphate exchange reaction in the presence of a wide variety of commerical amino acids which had been purified by recrystallization. For example, as seen in Table I, similar values of k_{cat} were found for methionine, isoleucine, valine, and α-aminobutyrate, although the K_M values are much higher for the nonspecific amino acids. It was suspected that these values were artifactual, being caused by trace impurities of methionine. On treatment of isoleucine and valine with Raney nickel to remove any methionine, all activity was lost.

[1] Abbreviations used: 3′-deoxy-tRNA, tRNA in which the terminal adenosine is replaced by 3′-deoxyadenosine; Nva, norvaline; Nle, norleucine; HCy, homocysteine.

Table I: Effect of Trace Impurities of Methionine in L-Amino Acids on Pyrophosphate Exchange Activity[a]

	Michaelis-Menten quantities[b]						
	before Raney nickel treatment			after Raney nickel treatment			
amino acid	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})	% Met in amino acid
methionine	10	0.047	2.1×10^5				100
isoleucine	8.8	17	520	0		0	0.24
valine	9.0	21	430	0		0	0.20
norvaline			14			17	0
α-aminobutyrate	8.1	188	43			0.28	0.02
alanine			0.4			0.04	2×10^{-4}
glycine			0.04			<0.01	2×10^{-7}

[a] Pyrophosphate exchange reaction at 25 °C, standard pH 7.78 buffer, 2 mM ATP and 2 mM [^{32}P]pyrophosphate catalyzed by the methionyl-tRNA synthetase from *B. stearothermophilus*. [b] Rate constants refer to moles of substrate per mole of enzyme dimer.

Table II: Activation of L-Amino Acids Catalyzed by the Methionyl-tRNA Synthetase from *B. stearothermophilus*[a]

substrate (concn range, mM)	side chain	k_{cat}[b] (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})	$\Delta\Delta G$[c] (kcal mol^{-1})
ethionine (0.1–10)	$CH_3CH_2SCH_2CH_2$	15	2.04	7.34×10^3	2.00
methionine (0.01–1)	$CH_3SCH_2CH_2$	10	0.047	2.13×10^5	
norleucine (0.125–20)	$CH_3CH_2CH_2CH_2$	4.9	4.7	1.04×10^3	3.15
homocysteine (0.5–100)	$HSCH_2CH_2$	4.0	1.78	2.7×10^3	2.59
norvaline[d] (10–165)	$CH_3CH_2CH_2$			14	5.70
α-aminobutyrate[d] (7.5–175)	CH_3CH_2			0.28	8.02
alanine[d] (50–550)	CH_3			0.04	9.17
glycine[d] (75–800)	H			<0.01	>10

[a] Pyrophosphate exchange reaction at 25 °C, standard pH 7.78 buffer, 2 mM ATP and 2 mM [^{32}P]pyrophosphate. [b] Moles of substrate per mole of enzyme dimer. [c] Binding energy relative to methionine, calculated from the ratios of k_{cat}/K_M (Fersht, 1977a). [d] Saturation kinetics not observed in accessible concentration range.

A considerable diminution was found with α-aminobutyrate, whilst the results with norvaline were little affected by this treatment (Table I). Addition of 2 mM methionine to reaction mixtures containing nearly saturating concentrations of the treated isoleucine, valine, and α-aminobutyrate restored the values of k_{cat} (~10 s^{-1}). The fractions of methionine present in the supposedly purified samples of amino acid required to give the observed pyrophosphate exchange activities prior to treatment with Raney nickel are given in Table I.

The pyrophosphate exchange activities with methionine-free amino acid preparations are summarized in Table II. Ethionine and homocysteine cannot be purified by the Raney nickel treatment as they would be desulfurized. However, the observed values of k_{cat} found in their presence are different from that found with methionine and are thus real. The activity with alanine is not caused by the presence of the alanyl-tRNA synthetase since the value of K_M is too high.

The traces of methionine in the natural amino acids arise as a consequence of their isolation from protein hydrolysates. α-Aminobutyrate is made commercially by the treatment of methionine with Raney nickel, hence the impurity (information courtesy of Calbiochem).

b. Aminoacylation Activity. It is seen in Table III that significant aminoacylation of tRNA was found only with ethionine, methionine, and norleucine. At the concentrations of [^{35}S]homocysteine and α-[^{14}C]aminobutyrate used, there is no buildup of labeled mischarged tRNA even when Tu–GTP is added to the reaction mixtures with the enzyme from *E. coli*. However, it will be seen later that the preparation of Tu–GTP used gives only partial protection against the enzyme-catalyzed deacylation.

c. Attempts to Mischarge tRNA^{Met} Preparatively. Incubating tRNA^{Met} (5–11 μM) with methionyl-tRNA synthetase (90–180 nM) in the presence of α-[^{3}H]But (45 mM) under aminoacylation conditions in the pH 7.78 buffer at 37 °C (when the pH decreases to about pH 7.5) led in general to no appreciable accumulation of charged tRNA.

Table III: Aminoacylation of tRNA^{Met} with Labeled Amino Acids[a]

substrate and source of methionyl-tRNA synthetase	concn (mM)	$[V_0]/[E]$[b] (s^{-1})
B. stearothermophilus		
ethionine	1.5	0.6[c]
methionine	saturating	1.6
norleucine	saturating	0.22[d]
homocysteine	0.16	<0.01
α-aminobutyrate	0.1	$<10^{-4}$
E. coli		
methionine	0.13	6.2
norleucine	0.8	0.4
homocysteine	1.2[e]	$<10^{-3}$
α-aminobutyrate	0.13[e]	$<10^{-6}$

[a] Standard pH 7.78 buffer, 2 mM ATP. [b] 30 nM to 1 μM enzyme, 13 μM tRNA, 1 unit/mL of inorganic pyrophosphatase. [b] Moles of substrate/mole of enzyme dimer for *B. stearothermophilus*. [c] Saturation kinetics not observed up to 1.5 mM substrate $k_{cat}/K_M = 400$ s^{-1} M^{-1}. [d] K_M = 1mM. [e] Plus 2–8 μM Tu-GTP, 5 mM phosphoenol pyruvate, 0.2 mM GTP, and pyruvate kinase. Similar results obtained with the proteolytic fragment.

Typically, less than 5–10% was found to be charged. Repeating the experiments replacing the α-aminobutyrate with [^{35}S]Met gave 100% aminoacylation of the tRNA. Also, replacing the tRNA by 3′-deoxy-tRNA^{Met} (*E. coli*) gave fully charged α-[^{3}H]But-3′-deoxy-tRNA^{Met}.

However, one batch of methionyl-tRNA synthetase from *E. coli* that had been stored frozen in aqueous solution in liquid nitrogen for nearly a year and had been repeatedly frozen and thawed successfully charged tRNA^{Met} with α-[^{3}H]But under the above conditions. This provided samples of α-[^{3}H]But-tRNA^{Met} which were used below for measurements of the enzyme-catalyzed deacylation rates. Amino acid analysis of the hydrolyzed product confirmed that the attached amino acid was α-aminobutyrate. Examination of the "aged" sample of enzyme by polyacrylamide gel electrophoresis in the presence

Table IV: ATP-Pyrophosphatase Activities[a]

amino acid	concn (mM)	$[V_o]/[E]$[b] (s^{-1})
B. stearothermophilus[c]		
ethionine	70	0.09
methionine	1	0.08
norleucine	40	0.23[d]
homocysteine	10	2.1
norvaline	105	0.4
α-aminobutyrate	75–400	0.07
E. coli[e]		
norleucine	40	1.2
norleucine	40	0.6[f]
norleucine	40	0.9[g]
homocysteine	10	4.4
α-aminobutyrate	312	0.1

[a] Standard pH 7.78 buffer, 2 mM ATP, indicated concentrations of $tRNA^{Met}$, 1 unit/mL of inorganic pyrophosphatase. [b] Moles of substrate/mole of enzyme dimer. [c] 2 μM $tRNA^{Met}$ (partly fractionated mixture of $tRNA^{Met}_f$ and $tRNA^{Met}_M$). [d] k_{cat} = 0.23, K_M ~1.8 mM. [e] 12 μM $tRNA^{Met}$. [f] Proteolytic fragment of enzyme used. Result approximate as active-site titration not performed. [g] 3′-Deoxy-$tRNA^{Met}$ (7 μM) replaced the $tRNA^{Met}$.

of sodium dodecyl sulfate revealed no apparent proteolysis.

No formation of [^{14}C]Nva-$tRNA^{Met}$ (*E. coli*) (<5 pmol/A_{260}) could be detected using 3 mM [^{14}C]Nva with either fresh or aged enzyme or when 3′-deoxy-$tRNA^{Met}$ was substituted for the $tRNA^{Met}$.

d. ATP–Pyrophosphatase Activity. It is seen in Table IV that there is a gradation of ATP–pyrophosphatase activity, peaking with homocysteine. The turnover number for the ATP–pyrophosphatase reaction of the enzyme from *B. stearothermophilus* in the presence of homocysteine and $tRNA^{Met}$ (a mixture of $tRNA^{Met}_M$ and $tRNA^{Met}_F$ in this case) is about 2.1 s^{-1}. This is very close to the turnover number of 2.4 s^{-1} for aminoacylation with methionine under these conditions with this sample of tRNA. Similarly, the rate constant of 4.4 s^{-1} for the homocysteine-dependent ATP-pyrophosphatase reaction of the enzyme from *E. coli* is similar to the aminoacylation rate constant of 6.2 s^{-1} (Table III). As it has been shown that the turnover number for aminoacylation is close to the rate constant for the transfer of methionine from the enzyme-bound methionyl adenylate complex to tRNA for the *B. stearothermophilus* system (Mulvey & Fersht, 1978), the kinetics are consistent with the following reaction:

$$E\cdot HCy\cdot ATP\cdot tRNA \xrightarrow{\text{fast}} E\cdot HCy\text{-}AMP\cdot tRNA \xrightarrow{\text{slow}} \begin{matrix} E\cdot HCy\text{-}tRNA + AMP \\ \downarrow \text{fast} \\ E + HCy + tRNA \end{matrix} \quad (1)$$

$$\downarrow PP_i$$

However, as seen below, there is no direct evidence for the transient formation of the mischarged tRNA.

Significant ATP–pyrophosphatase activity is found also in the presence of norleucine and norvaline. This rules out the possibility that the activity in the presence of homocysteine is just an artifact of the well-known propensity of homocysteinyl derivatives to cyclize.

Substitution of 3′-deoxy-$tRNA^{Met}$ for $tRNA^{Met}$ in the norleucine-dependent reaction decreases the ATP–pyrophosphatase activity by only 25%. The 3′-OH group of the terminal adenosine is thus not essential for the hydrolytic activity here.

Table V: Methionyl-tRNA Synthetase Catalyzed Deacylation of aa-$tRNA^{Met}$

enzyme source	aa	additions	k_{obsd}[a] (s^{-1})
B. stearothermophilus	Nle		9.1×10^{-3}
	Nle	Nle-AMP	5.3×10^{-3}
E. coli	Nle		1.8×10^{-2}
	Nle	Tu–GTP	4.5×10^{-3}
	αBut		9.5×10^{-3}
	αBut[b]		8.7×10^{-3}

[a] 25 °C, pH 7.78 standard buffer. Single turnover conditions of ~10 μM enzyme and 1 μM aa-$tRNA^{Met}$. [b] 3′-deoxy-tRNA deacylated.

e. Dual Aminoacylation and ATP–Pyrophosphatase Activities in the Presence of Norleucine. The finding that the methionyl-tRNA synthetases catalyze both the aminoacylation of tRNA with norleucine *and* simultaneously an ATP–pyrophosphatase activity under identical conditions was most surprising. The reactions were studied in detail for the enzyme and tRNA from *B. stearothermophilus*. The two processes have a very similar value for k_{cat}, about 0.22 s^{-1}, and similar values of K_M, about 1–2 mM. The dual activities are not due to inhomogeneities in the population of tRNA. On incubating a mixture of [^{3}H]norleucine (0.8 mM), methionyl-tRNA synthetase (1 μM), ATP (2 mM), inorganic pyrophosphatase (1 unit/mL), and either $tRNA^{Met}_F$ or $tRNA^{Met}_M$ (both 15 μM), both samples of tRNA were found to be aminoacylated at 71% of the maximum found with methionine. The $tRNA^{Met}$ from *E. coli* could be aminoacylated to 54% of the maximum.

An enzyme-bound norleucinyl adenylate complex could be isolated by gel filtration and a typical fraction of the activity transferred to tRNA. For example, on incubating a mixture of methionyl-tRNA synthetase (*B. stearothermophilus*, 15 μM), [^{3}H]norleucine (0.8 mM), ATP (1 mM), and inorganic pyrophosphatase (1 unit/mL) for 1 min at 25 °C, followed by gel filtration on Sephadex G-25 at 4 °C in the standard pH 5.87 buffer, an enzyme-bound aminoacyl adenylate complex of stoichiometry 0.8–1.1 was obtained. On addition of $tRNA^{Met}_F$, 66–90% of the radioactivity was found to be transferred to the tRNA.

f. Methionyl-tRNA Synthetase Catalyzed Deacylation of aa-$tRNA^{Met}$. These experiments were conducted to determine whether the editing occurs by the deacylation of the mischarged tRNA. It is seen in Table V that Nle-$tRNA^{Met}$ and αBut-$tRNA^{Met}$ are relatively stable in the presence of excess enzyme. The addition of the Tu–GTP complex to the reaction mixture does not afford complete protection of Nle-$tRNA^{Met}$ against deacylation, slowing down the rate constant by a factor of 4 only. In comparison, Ile-$tRNA^{Ile}$ and Val-$tRNA^{Val}$ are considerably stabilized by the presence of Tu–GTP under similar conditions in the presence of the cognate aminoacyl-tRNA synthetases.

There is little difference in reactivity between αBut-$tRNA^{Met}$ and αBut-3′-deoxy-$tRNA^{Met}$.

g. Attempt to Detect Transiently Mischarged Homocysteinyl-$tRNA^{Met}$. The complex between homocysteinyl adenylate and the methionyl-tRNA synthetase from *E. coli* was not sufficiently stable to be isolated by gel filtration and so it was prepared in situ and its concentration determined by nitrocellulose disk filtration. (Millipore rather than Schleicher & Schull filters were used for this case as the latter gave abnormally high retention of radioactivity.) On mixing with $tRNA^{Met}$ in the quenched-flow apparatus, less than 2.5% of the radioactivity was found to be transferred to tRNA in the time range of 8–160 ms. However, as we were never able to

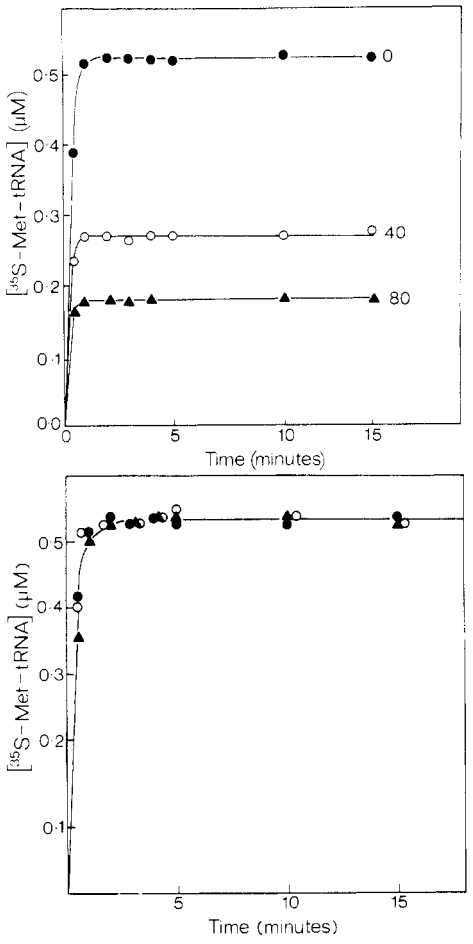

FIGURE 1: Depression of formation of [^{35}S]Met-tRNA^{Met} in an aminoacylation mixture on addition of α-aminobutyrate. (Upper) Commercial α-aminobutyrate (40 mM, 0: 80 mM, ▲) was added to a solution of [^{35}S]Met (2.7, μM), ATP (2 mM), tRNA^{Met} (0.51 μM), methionyl-tRNA synthetase from *E. coli* (36 nm) and inorganic pyrophosphatase (0.5 unit/mL) in the standard pH 7.78 buffer as described in the text. (Lower) Repetition of above using α-aminobutyrate that had been freed from methionine by Raney nickel.

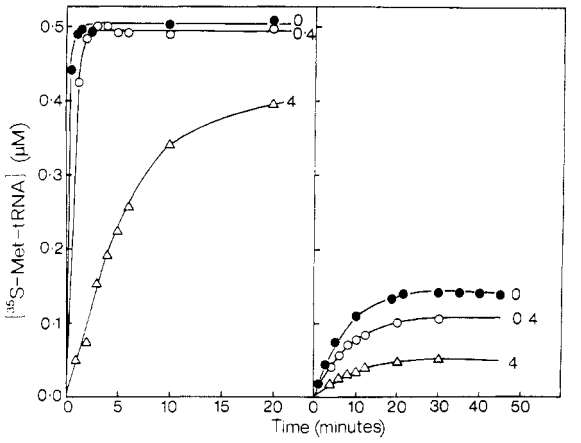

FIGURE 2: Depression of formation of [^{35}S]Met-tRNA^{Met} in an aminoacylation mixture on addition of DL-methioninol. (Left) 0.4 mM (O) or 4 mM (Δ) added as in Figure 1. (Right) As on the left but in the presence of 25 mM ATP and 25 mM $MgCl_2$.

isolate homocysteinyl-tRNA^{Met}, there is no knowledge of its stability in the presence of trichloroacetic acid. All manipulations of precipitation and filtration were performed as rapidly as possible.

h. Concerning the Depression of the Formation of Met-tRNA^{Met} by Added Amino Acids. The previous evidence for the mischarging of tRNA^{Met} was based on measurements of the depression of the formation of [^{14}C]Met-tRNA^{Met} in an aminoacylation mixture containing known amounts of [^{14}C]Met and an unlabeled competing amino acid (Old & Jones, 1976, 1977). An example of this is illustrated in Figure 1 where it is seen that the addition of commercial α-aminobutyrate depresses the amount of tRNA^{Met} charged with [^{14}C]Met. It was assumed previously that this depression is due to the accumulation of α-aminobutyryl-tRNA^{Met}. However, the depression is caused by the impurity of methionine in the α-aminobutyrate diluting out the labeled methionine. There is no depression when samples of methionine-free α-aminobutyrate are used (Figure 1). There is a further reason why the method of Old & Jones (1976, 1977) can give artifactual results. In an aminoacylation mixture, the amount of tRNA that is charged is always at a steady-state plateau representing the balance between synthesis and hydrolysis. The addition of a competitive amino acid will lower the rate of synthesis by simple competition for the active site of the enzyme and hence lower the steady-state plateau. This is illustrated in Figure 2 where methioninol, a nonesterifiable analogue of methionine, is seen also to depress the formation of [^{14}C]Met-tRNA^{Met}. This is not due to impurities of unlabeled methionine in the methioninol since it did not support the pyrophosphate exchange reaction at a concentration of 4–14 mM in the presence of 1 μM enzyme. The depression is more marked at the higher concentration of ATP (25 mM) used by Old & Jones (1977) where the aminoacylation rate is lower (Figure 2, right-hand panel).

Discussion

Trace Impurities of Methionine Obscure the Pattern of Misactivations and Conceal the Editing Mechanism. It has previously been reported that the methionyl-tRNA synthetase from *E. coli* catalyzes the pyrophosphate exchange reaction in the presence of a wide variety of amino acids with unbranched side chains (Old & Jones, 1977). The value of k_{cat} remained constant, close to that found for the reaction with methionine. The same was found in this study for the enzyme from *B. stearothermophilus* when recrystallized samples of commercial amino acids were used (Table I). However, following treatment with Raney nickel to remove any contamination with methionine, the pattern of activities changes dramatically. As seen in Table II, the value of k_{cat}/K_M decreases monotonically as the length of the side chain is reduced below that of methionine, whilst the values of K_M for norvaline, α-aminobutyrate, and alanine are too high to be measured.

The presence of methionine as a contaminant also concealed the presence of an editing mechanism for the reasons described in the previous section. There is an editing mechanism for removing the misactivation of smaller amino acids. The evidence for this is that these amino acids are activated by the enzymes (the presence of the amino acid dependent pyrophosphate exchange reaction, Table II) but they are not transferred to tRNA^{Met} (Table III). Instead, they stimulate an ATP–pyrophosphatase activity in the presence of tRNA^{Met}, showing that the amino acid is being continuously activated but the products are hydrolyzed somewhere along the reaction pathway.

Editing Mechanism Sorts the Amino Acids According to Size. Ethionine, which is larger than methionine, is successfully transferred to tRNA^{Met}, whilst the smaller amino

acids only are edited. Norleucine, which has the same number of heavy atoms in the side chain as methionine, is on the borderline of being edited; it is both transferred to tRNA and stimulates an ATP–pyrophosphatase activity. This must reflect that norleucine is slightly smaller than methionine, the C–C bond being about 0.4 Å shorter than the C–S bond.

This pattern of behavior is in accord with the "double-sieve" model for editing (Fersht, 1977a; Fersht & Dingwall, unpublished experiments). In this model, there are two active sites, one for synthesis and one for hydrolysis. It is considered that the basic problem in specificity is the rejection of naturally occurring amino acids that are isosteric with or smaller than the specific amino acid. The activation of larger amino acids may be kept down to an insignificant level by steric hindrance of their binding, as has been found for the activation of isoleucine by the valyl-tRNA synthetase (Fersht and Dingwall, unpublished experiments), but the mistaken activation of isosteric and smaller amino acids must often be corrected by hydrolytic editing. Extending this model to amino acids that do not occur naturally, it is expected that (a) smaller unnatural amino acids will be activated and their reaction products edited in the same way as for smaller naturally occurring acids since selection is on size; (b) larger amino acids may be activated since the enzyme has not needed to evolve to discriminate against the particular stereochemistry, and, if so, the products will not be edited at an appreciable rate since the substrate will be too large to be accommodated in the hydrolytic site; (c) unnatural isosteres will also be activated and probably escape the editing reaction since the enzyme has not evolved to recognize the specific features of the unnatural isostere. (For example, the valyl-tRNA synthetase recognizes the hydroxyl group of threonine to draw it into the hydrolytic site, but α-chloro-β-aminobutyrate escapes being edited.)

The target of the editing reaction of the methionyl-tRNA synthetases is presumably homocysteine. The editing of the other smaller amino acids is just a consequence of the selection by size.

Binding Energy of the Side Chain of Methionine. The contribution of the side chain to the binding energy may be calculated from the value of k_{cat}/K_M for the activation reaction. The data are listed in Table II. Comparing the values for homocysteine and α-aminobutyrate, it is seen that the sulfur atom contributes a binding energy of 5.43 kcal/mol. Similarly, comparing the values for methionine and norleucine, or homocysteine and norvaline, it is seen that the sulfur atom contributes 3.11–3.15 kcal/mol of binding energy relative to the CH_2 group.

Nonaccepting Terminal Hydroxyl Group Is Not Essential for Editing. The involvement of the nonaccepting hydroxyl group of the terminal adenosine of tRNA in the editing mechanism stems from the observation by von der Haar & Cramer (1975) that 3′-deoxy-tRNA^{Ile} may be mischarged with valine. Our results show that this is not a general phenomenon. Although 3′-deoxy-tRNA^{Met} may be charged with methionine, it is not mischarged with norvaline or homocysteine. Further, the norleucine-stimulated ATP–pyrophosphatase activity of the methionyl-tRNA synthetase from *E. coli* in the presence of 3′-deoxy-tRNA^{Met} is 75% of that in the presence of tRNA^{Met} (Table IV). Combined with the observations that 3′-deoxy-tRNA^{Val} (yeast) may be charged to only 50% with threonine (Igloi et al., 1977) and that 3′-deoxy-tRNA^{Met} may be mischarged with α-aminobutyrate, it would appear that the nonaccepting hydroxyl plays some role in the editing reaction, but the presence of the hydroxyl is not crucial. This appears to rule out the mechanism of von der Haar & Cramer (1976) and Igloi et al. (1977) which involves the catalytic participation of the nonaccepting OH group. It is not inconsistent with the proposal of Fersht & Kaethner (1976) that editing requires a translocation of the aminoacyl moiety to the hydrolytic site by either a movement of the terminal adenosine of the tRNA or by an intramolecular 2′ → 3′ OH acyl transfer.

Problem of the Chemical Pathway of Editing. It is not clear whether the editing reaction occurs before the transfer of amino acid to tRNA^{Met} or by the deacylation of the mischarged tRNA. Just as for the rejection of valine by the isoleucyl-tRNA synthetase (Fersht, 1977b), the kinetics appear at first sight to be consistent with the slow formation of mischarged tRNA, followed by its rapid enzyme-catalyzed hydrolysis. For example, the turnover number for the homocysteine-stimulated ATP–pyrophosphatase activity of the enzyme from *B. stearothermophilus* is very similar to the rate constant for the transfer of methionine from the E·Met–AMP complex to tRNA^{Met}. The logical inference is that the ATP–pyrophosphatase reaction involves the rate-determining misacylation of the tRNA, followed by its rapid hydrolysis. However, just as for the rejection of valine by the isoleucyl-tRNA synthetase, the transiently mischarged tRNA could not be detected. Further, in the case of norleucine, the mischarged tRNA was isolated and found to be hydrolyzed far too slowly to account for the observed rate of the ATP–pyrophosphatase reaction. Various explanations of the type of behavior have been put forward previously (Fersht, 1977b). Now that the misacylation–deacylation pathway has been so clearly demonstrated for the valyl-tRNA synthetases (Fersht & Dingwall, 1979a), it is tempting to generalize this mechanism by hypothesizing that the enzyme is in a more reactive state for hydrolysis during the aminoacylation reaction than when the enzyme is added to preformed mischarged tRNA.

References

Baldwin, A. N., & Berg, P. (1966) *J. Biol. Chem. 241*, 839.

Cassio, D., & Waller, J. P. (1971) *Eur. J. Biochem. 20*, 283.

Fayat, G., Blanquet, S., Dessen, P., Batelier, P., & Waller, J-P. (1974) *Biochimie 56*, 35.

Fersht, A. R. (1977a) *Enzyme Structure and Mechanism*. Chapter 11, W. H. Freeman, San Francisco, CA.

Fersht, A. R. (1977b) *Biochemistry 16*, 1025.

Fersht, A. R., & Jakes, R. (1975) *Biochemistry 14*, 3350.

Fersht, A. R., & Kaethner, M. M. (1976) *Biochemistry 15*, 3342.

Fersht, A. R., & Dingwall, C. (1979a) *Biochemistry*, first paper of three in this issue.

Fersht, A. R., & Dingwall, C. (1979b) *Biochemistry*, preceding paper in this issue.

Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L. E., & Hartley, B. S. (1975) *Biochemistry 14*, 1.

Fonken, G. S., & Mozingo, R. (1947) *J. Am. Chem. Soc. 69*, 1212.

Igloi, G. L., von der Haar, F., & Cramer, F. (1977) *Biochemistry 16*, 1696.

Mulvey, R. S., & Fersht, A. R. (1976) *Biochemistry 15*, 243.

Mulvey, R. S., & Fersht, A. R. (1977) *Biochemistry 16*, 4731.

Mulvey, R. S., & Fersht, A. R. (1978) *Biochemistry 17*, 5591.

Old, J. M., & Jones, D. S. (1976) *Biochem. J. 159*, 503.

Old, J. M., & Jones, D. S. (1977) *Biochem. J. 165*, 367.

Sprinzl, M., & Cramer, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3049.

Tal, J., Deutscher, M. P., & Littauer, U. Z. (1972) *Eur. J. Biochem. 28*, 478.

Trupin, J., Dickerman, H., Nirenberg, M., & Weissbach, H. (1966) *Biochem. Biophys. Res. Commun. 24*, 50.
von der Haar, F., & Cramer, F. (1975) *FEBS Lett. 56*, 215.
von der Haar, F., & Cramer, F. (1976) *Biochemistry 16*, 4131.
Walker, J. E., Shaw, D. C., Northrop, F. D., & Horsenell, T. (1978) *Solid Phase Methods in Protein Sequencing Analysis* (Previero, E., & Coletti-Previero, M. A., Eds.) p 69, North-Holland Publishing Co., Amsterdam.

Side-Chain Torsional Potentials: Effect of Dipeptide, Protein, and Solvent Environment†

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ABSTRACT: Side-chain torsional potentials in the bovine pancreatic trypsin inhibitor are calculated from empirical energy functions by use of the known X-ray structure of the protein and the rigid-geometry mapping technique. The potentials are analyzed to determine the roles and relative importance of contributions from the dipeptide backbone, the protein, and the crystalline environment of solvent and other protein molecules. The structural characteristics of the side chains determine two major patterns of energy surfaces, $E(\chi_1,\chi_2)$: a γ-branched pattern and a pattern for longer, straight side chains (Arg, Lys, Glu, and Met). Most of the dipeptide potential curves and surfaces have a local minimum corresponding to the side-chain torsional angles in the X-ray structure. Addition of the protein forces sharpens and/or selects from these minima, providing very good agreement with the experimental conformation for most side chains at the surface or in the core of the protein. Inclusion of the crystalline environment produces still better results, especially for the side chains extending away from the protein. The results are discussed in terms of the details of the interactions due to the surrounding, calculated solvent-accessibility figures and the temperature factors derived from the crystallographic refinement of the pancreatic trypsin inhibitor.

Although much progress has been made in determining the factors responsible for the equilibrium conformation of a protein (Richards, 1977; Chothia et al., 1977; Anfinsen & Scheraga, 1975), a detailed understanding has not yet been achieved. One important question concerns the interactions that hold a given part of a protein in the position observed in the native structure. Since fluctuations relative to the native structure do occur, it is also of interest to evaluate the restoring forces which arise upon small displacement from equilibrium. This paper is concerned with an empirical energy function analysis of the interactions which determine the side-chain torsional potentials, given the conformation of a protein. We examine all the side chains of a single protein, the bovine pancreatic trypsin inhibitor (PTI), and focus on the relative importance of the energy contributions due to short-range (side-chain, backbone) interactions, interactions with the rest of the protein, and interactions with solvent and neighboring protein molecules of the crystalline environment.

A complementary approach to side-chain orientations is a statistical treatment of the torsional angles observed in series of proteins. Such an analysis has been made by Chandrasekaran & Ramachandran (1970). They used contact criteria for a dipeptide to determine the allowed side-chain torsional angles. Estimates of the relative probabilities of different side-chain orientations were made and found to agree well in an overall sense with the observed distributions. No attempt was made to examine individual cases nor to evaluate interactions beyond the dipeptide (i.e., from other parts of the protein and the surroundings).

The bovine pancreatic trypsin inhibitor is used in the present study for several reasons. Its X-ray crystal structure has been determined to high resolution (Deisenhofer & Steigemann, 1975) and shows many localized solvent molecules, whose steric effects on side-chain conformation can be evaluated. The small size of PTI (58 residues, 454 nonhydrogen atoms) has made possible extensive calculations including energy minimization (Gelin & Karplus, 1975), a study of aromatic side-chain rotations ("ring flips") (Gelin & Karplus, 1975; Snyder et al., 1975; Hetzel et al., 1976), and a molecular dynamics calculation of the fluctuations about the equilibrium conformation (McCammon et al., 1977).

The method used for calculating protein conformational energy and the procedures for evaluating the various contributions to side-chain torsional potentials are described under Methods. The results of the calculations are presented under Results along with their structural interpretations and comparisons with the observed torsional angles. In the final section the results are discussed and related to measures of solvent accessibility and thermal mobility of different parts of the protein.

Methods

The energy function and protein structure definition are outlined below, followed by a description of the rigid mapping procedure used to determine the side-chain torsional potentials.

A. Energy Function and Protein Structure Definition. The conformational energy of a macromolecule in vacuo or in surroundings representing the crystal environment has been calculated using empirical energy functions. The functional forms chosen for the various types of interactions, the methods of specification of the covalent structure of the protein, and the evaluation and application of the resulting energy expression are outlined below.

The potential energy is written as a sum of terms, one set of which arises from the isolated protein and another from the interactions of the protein with its environment (solvent and other proteins) in the crystal. The isolated protein terms correspond to bonds, bond angles, torsional angles, van der Waals interactions, electrostatic interactions, and hydrogen

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