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Isoleucyl-tRNA Synthetase from Bakers' Yeast: Multistep Proofreading in Discrimination between Isoleucine and Valine with Modulated Accuracy, a Scheme for Molecular Recognition by Energy Dissipation

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ABSTRACT: For discrimination between isoleucine and valine by isoleucyl-tRNA synthetase from yeast, a multistep sequence is established. The initial discrimination of the substrates is followed by a pretransfer and a posttransfer hydrolytic proofreading process. The overall discrimination factor D was determined from k_{cat} and K_m values observed in aminoacylation of $tRNA^{Ile}$-C-C-A with isoleucine and valine. From aminoacylation of the modified tRNA species $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-A(3′NH_2), the initial discrimination factor I (valid for the reversible substrate binding) and the proofreading factor P_1 (valid for the aminoacyl adenylate formation) could be determined. Factor I was computed from ATP consumption and D_1, the overall discrimination factor for this partial reaction which can be obtained from kinetic constants, and P_1 was calculated from AMP formation rates. Proofreading factor P_2 (valid for aminoacyl transfer reaction) was determined from AMP formation rates observed in aminoacylation of $tRNA^{Ile}$-C-C-A and $tRNA^{Ile}$-C-C-3′dA. From the initial discrimination factor I and the AMP formation rates, discrimination factor D_{AMP} in aminoacylation of $tRNA^{Ile}$-C-C-A can be calculated. These values deviate by a factor Π from factor D obtained by kinetics which may be due to the fact that for acylation of $tRNA^{Ile}$-C-C-A an initial discrimination factor $I' = I\Pi$ is valid. The observed overall discrimination varies up to a factor of 16 according to conditions. Under optimal conditions, 38 000 correct aminoacyl-tRNAs are produced per 1 error while the energy of 5.5 ATPs is dissipated. With the determined energetic and molecular flows for the various steps of the enzymatic reaction, a coherent picture of this new type of "far away from equilibrium enzyme" emerges.

The frequency by which valine may substitute for isoleucine in vivo has been determined in direct measurements to be about 1:3000 (Loftfield, 1963; Loftfield & Vanderjagt, 1972). To achieve this high specificity of protein biosynthesis, all enzymes involved in the translational process should exhibit the same specificity since the step with the lowest accuracy determines the overall fidelity.

A first conjecture about the possible accuracy which may be achieved by an enzyme in discrimination between isoleucine and valine was made by Pauling (1958). Starting from the assumption that the smaller amino acid valine should also fit into the binding site of isoleucine, Pauling calculated the van der Waals attraction energy with which the additional methylene group of isoleucine might interact with the enzyme surface. According to the London theory of electronic dispersion forces, a value of about 0.9 kcal/mol (3.77 kJ/mol) was found which corresponds to a discrimination factor of 4.3 in favor of isoleucine. This value was confirmed by measurements of the interaction of methyl-substituted antigens with antibodies (Pauling & Pressman, 1945).

In a similar calculation, Hopfield & Yamane (1979) estimated the optimal difference in binding energy between isoleucine and valine to be about 2.5 kcal/mol (10.46 kJ/mol, corresponding to a discrimination of 1:60) on the basis of the assumption that the critical methyl group of isoleucine might be surrounded by 6–9 methyl equiv.

To obtain directly the discrimination factor caused by different binding energies, the kinetic constants k_{cat} and K_m were measured for both substrates in the ATP/PP_i pyrophosphate exchange reaction which represents the reverse of the first reaction step of the aminoacylation reaction:

$$E^{Ile} + aa + ATP \rightleftharpoons E^{Ile}\cdot aa\text{-}AMP + PP_i$$

$$E^{Ile}\cdot aa\text{-}AMP + tRNA^{Ile} \rightleftharpoons E^{Ile} + aa\text{-}tRNA^{Ile} + AMP$$

For the discrimination factor $D_{PP_i} = (k_{cat}/K_m)_{Ile}(k_{cat}/K_m)_{Val}^{-1}$, values of about 200 were calculated due to a difference of about 3 kcal/mol (12.6 kJ/mol) in the free binding enthalpies of the two substrates (Fersht, 1977a, b, 1981; Fersht et al., 1980). In a different approach, $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-A(3′NH_2) were aminoacylated with isoleucine and valine, and from k_{cat} and K_m values, similar discrimination factors were obtained (Cramer et al., 1979; Freist & Cramer, 1983). These discrimination factors were considered to be due to the binding energy of the additional methylene group of isoleucine.

Because the discrimination factors mentioned above are too low to explain the high in vivo accuracy, the enzyme must act

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with a correction mechanism which rejects valine (Hopfield et al., 1976; Fersht, 1977a,b, 1981; von der Haar & Cramer, 1976). For quantitative investigations of this "proofreading" mechanism, the stoichiometry of AMP formation was measured, and values in the range of 200–800 mol of AMP for 1 mol of misacylated tRNA were reported (Cramer et al., 1979; Hopfield et al., 1976). The resulting estimates of error rates were ranging from 1:10000 to 1:180000 (Hopfield et al., 1976; Cramer et al., 1979). However, more exact values of error rates are desirable for judgement of the error catastrophe theory, which is one of the major current theories of aging of cells (Bullough, 1971; Orgel, 1963, 1973; Hayflick, 1975).

In the present studies, we investigate the overall discrimination between isoleucine and valine in aminoacylation of $tRNA^{Ile}$ with isoleucyl-tRNA synthetase from yeast for the first time under different assay conditions which may be considered as a stepwise approximation to physiological conditions. The observed discrimination factors are in good accordance with the error frequencies determined in vivo. Furthermore, we are able to draw new conclusions on energy differences in the binding of isoleucine and valine and to show that the enzyme acts by a more complicated proofreading mechanism than has been assumed up till now, coupling the molecular flow of amino acids to an energetical flow of ATP hydrolysis.

Experimental Procedures

Materials. Isoleucyl-tRNA synthetase (EC 6.1.1.5) was purified from bakers' yeast according to von der Haar (1973); the preparation had a specific activity of 493 units/mg of protein in the aminoacylation reaction. One enzyme unit catalyzed the aminoacylation of 1 nmol of tRNA in 1 min at 37 °C under standard conditions. For determination of k_{cat} values, the concentration of active enzyme was determined by the nitrocellulose filter assay according to Fersht & Kaethner (1976a) before the experiments were run. The absence of valyl-tRNA synthetase in the isoleucyl-tRNA synthetase preparation was proven by addition of valine and $tRNA^{Val}$ in an aminoacylation assay, whereupon only background counts were observed.

Pure $tRNA^{Ile}_{II}$ was isolated from commercially available yeast tRNA (Boehringer Mannheim, FRG) as described by von der Haar & Cramer (1978). The $tRNA^{Ile}_{II}$ preparation was tested with valine and valyl-tRNA synthetase to prove the absence of any valine-accepting tRNA species, yielding only background counts. Furthermore, the preparation was analyzed by high-pressure liquid chromatography (HPLC), applying the method described by Bischoff et al. (1983), and again found to be free of other tRNA species. The modified tRNAs $tRNA^{Ile}$-C-C-A(3′NH_2), $tRNA^{Ile}$-C-C-3′dA, and $tRNA^{Ile}$-C-C-2′dA were prepared with the aid of nucleotidyltransferase by reaction of 2′-dATP, 3′-dATP, and 3′-deoxy-3′-amino-ATP with $tRNA^{Ile}$-C-C and analyzed as described by Sprinzl et al. (1977), Cramer et al. (1975), Maelicke et al. (1974), Fraser & Rich (1973), and Sternbach et al. (1971). The preparations were also controlled by HPLC (Bischoff et al., 1983) and found to be free of $tRNA^{Ile}$-C-C-A or other compounds.

^{14}C-Labeled isoleucine and ^{14}C-labeled valine with specific activities of 342 and 275 mCi/mmol were purchased from the Radiochemical Centre (Amersham, Great Britain). The valine preparation was analyzed for traces of isoleucine by two-dimensional thin-layer chromatography on cellulose F aluminum sheets (Merck, Darmstadt, FRG) with 1-butanol/acetic acid/water (12:3:5) as the solvent system. Unlabeled valine and isoleucine were added to the sample, spots were made visible by ninhydrin spray reagent (Merck, Darmstadt, FRG) and cut out, and the radioactivity was counted in a liquid scintillation counter. The content of isoleucine in the valine preparation was determined to be about 0.01%. The same value was calculated from aminoacylation kinetics obtained in aminoacylation of $tRNA^{Ile}$-C-C-A with the commercial [^{14}C]valine preparation. In the first minutes of the aminoacylation reaction, a very small burst phenomenon was observed which is followed for the next 30–40 min by a small linear increase of reaction product [compare also Hopfield et al. (1976)]. The size of the small burst corresponds to 0.01% of the total amino acids of the reaction mixture. The origin of the small burst phenomenon was proven by a control experiment in which 0.01% [^{14}C]isoleucine was added to unlabeled valine and this mixture was used in an aminoacylation experiment. Only the small burst was observed without the following slightly increasing part of kinetics observed with the commercial [^{14}C]valine preparation. To assure that the isoleucine contamination of 0.01% does not falsify the valylation rates observed in the slightly increasing parts of misacylation kinetics, increasing amounts of [^{14}C]isoleucine were added to the [^{14}C]valine preparation, and the slopes of the misacylation reaction kinetics were compared. Additional amounts of 0.01–0.04% [^{14}C]isoleucine did not change misacylation rates within the limits of error which are in the range of ±15% for the slopes of misacylation kinetics. Because the small isoleucine contamination does not falsify measurements of misacylation kinetics, the commercial [^{14}C]valine preparation was used without purification. The concentration of isoleucine in the reaction mixture is in the very low range of 1/30000–1/100000 of its K_m value in the presence of a 10000-fold excess of valine.

Elongation factor Tu (EF-Tu) was isolated as described in Freist & Sternbach (1984). Inorganic pyrophosphatase (EC 3.6.1.1) with a specific activity of 635 units/mg of protein was obtained from Sigma Chemical Co. (St. Louis, MO). Pyruvate kinase from rabbit muscle, phosphoenolpyruvate, guanosine 5′-triphosphate (GTP), and guanylyl imidodiphosphate (GMP-PNP) were products of Boehringer Mannheim (Mannheim, FRG). Spermine was obtained from Serva Feinbiochemica (Heidelberg, FRG). [8-^{14}C]Adenosine 5′-triphosphate with a specific activity of 546 mCi/mmol was purchased from Amersham Buchler GmbH (Braunschweig, FRG) and was diluted with a 10-fold amount of unlabeled material.

Aminoacylation of $tRNA^{Ile}$-C-C-A with Valine. The velocity of the esterification reaction was measured by the amount of [^{14}C]valine incorporated into tRNA approximately as described by Faulhammer & Cramer (1977). The standard reaction mixture (0.1 mL) contained 0.15 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.65, 0.1 M KCl, 0.015 M $MgSO_4$, 2 mM ATP, 6 μM $tRNA^{Ile}$, and valine concentrations varying in the range 30–70 μM. In experiments with inorganic pyrophosphatase, 0.25 unit of this enzyme was added to 0.1 mL of the reaction mixture. For kinetics in the presence of EF-Tu·GTP, this complex was regenerated from EF-Tu·GDP in a mixture consisting of 0.1 M Tris-HCl buffer, pH 7.65, 0.1 M KCl, 0.02 M $MgSO_4$, 0.8 mM phosphoenolpyruvate, 800 μg/mL pyruvate kinase, 0.08 mM GTP, and also 0.035 mM GMP-PNP and 0.015 mM EF-Tu·GDP by incubation at 37 °C for 10 min. The aminoacylation mixture contained a 25 μM portion of this EF-Tu·GTP regeneration solution per 0.1 mL. If spermine was present in the aminoacylation mixture, its concentration was

1 mM. Because the K_d values of spermine– and Mg^{2+}–ATP complexes are the same order of magnitude ($\sim 10^{-4}$ and 0.5×10^{-5}, respectively; Lövgren et al., 1978), in the presence of 15 mM Mg^{2+} and 1 mM spermine practically all ATP is present in the form of the Mg^{2+}–ATP complex. The effect of spermine may be due to complex formation with the tRNA (Lövgren et al., 1978). When the Mg^{2+} concentration is lowered to 1 mM, complexes of ATP with Mg^{2+} or spermine are present in similar amounts, and the effect of spermine may also be due to ATP–spermine complex formation. For aminoacylation at pH 9, the mixture consisted of 0.01 M Tris-HCl buffer, pH 9, 0.008 M $MgSO_4$, and the same concentrations of ATP, [^{14}C]valine, and tRNA as given above. Experiments at pH 6.50 were carried out in 0.15 M piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) (PIPES) buffer (Merck AG, Darmstadt, FRG). The reaction mixture was preincubated at 37 °C, and then the enzyme was added. Samples of 0.015 mL were taken in intervals of 10 min. The concentration of active enzyme was 30 pM.

For determination of misacylation rates, only the slightly increasing parts of the kinetics were considered. The slopes of this misacylation section were calculated statistically for different valine concentrations, and the resulting misacylation rates were used to determine K_m and k_{cat} values. To achieve an acceptable accuracy at the low reaction rates, each experiment was repeated up to 5 times, and for each experiment, the radioactivity incorporated into tRNA was counted 4 times in the liquid scintillation counter. All kinetic data for incorporation rates of valine into $tRNA^{Ile}$ at the single valine concentrations were treated by the computerized linear regression approximation. Furthermore, in experiments without addition of the EF-Tu·GTP complex for which very low reaction rates were observed, the obtained values for incorporation of ^{14}C-labeled amino acid into tRNA were tested for statistical significance by *F* and *t* tests (Ramm & Hofmann, 1982) and were found to be highly significant. K_m and k_{cat} values were determined from Lineweaver–Burk plots which were again calculated with computerized linear regression. For the final values of K_m and k_{cat}, first average values were calculated from the different countings of the single experiments, and with these data, average values from all experiments were determined. For the resulting discrimination factors for isoleucine against valine (Table I), an experimental error of ±30% is estimated.

Aminoacylation of $tRNA^{Ile}$-C-C-N with Isoleucine and Valine. Aminoacylation of $tRNA^{Ile}$-C-C-A(3′NH_2) and $tRNA^{Ile}$-C-C-3′dA was performed in the same buffer mixtures as given above with an ATP concentration of 0.5 mM, varying the isoleucine or valine concentration in the range of 1.5–7 μM. The concentration of $tRNA^{Ile}$-C-C-N was 0.2 μM in the aminoacylation with isoleucine and 0.6 μM with valine. Active enzyme was added to a concentration of 60 pM. Samples of 0.02 mL were taken in intervals of 0.5 min in isoleucylation and of 1 min in valylation, and all data were treated as in Freist & Cramer (1983).

Inorganic Pyrophosphatase Assay. Inorganic pyrophosphatase was assayed in 0.4 M Tris-HCl (pH 7.65) containing 20 mM $MgSO_4$ and 3 mM pyrophosphatase. Spots were analyzed by Hanes' reagent (Hanes & Isherwood, 1949).

AMP Formation under Aminoacylation Conditions. The assay mixture contained the same constituents as in the aminoacylation assay. The concentration of [^{14}C]ATP was 0.2 mM; of [^{14}C]isoleucine or [^{14}C]valine, 0.02 mM; of $tRNA^{Ile}$, 6 μM; and of $tRNA^{Ile}$-C-C-N, 0.2 μM. To measure the aminoacylation rates, samples of 0.005 mL were taken at intervals of 0.25 min in the case of isoleucine and 5 min in the case of valine and treated as in Faulhammer & Cramer (1977). For determination of the AMP formation rate, samples of 0.005 mL from the same reaction mixture were spotted onto poly(ethylenimine)–cellulose (PEI–cellulose) F sheets (Merck, Darmstadt, FRG) at intervals of 0.5 min with isoleucine and of 5 min with valine. Unlabeled ATP, ADP, and AMP were applied to the plates at the origin prior to use. The nucleotides were separated by ascending chromatography for 90 min with a mixture of 1 M LiCl in 1 M acetic acid and 2-propanol (2:1 v/v). After being dried, the spots were marked in UV light and cut out always in the same size. The radioactivity was counted always in the same type of vials in a liquid scintillation counter in a xylene-based scintillator (Scintillator 303, Riedel-De Haen, Hannover, FRG). The observed radioactivity was related to a standard measured also on a PEI–cellulose F piece of the same size as the AMP spots cut out. Each experiment was repeated up to 10 times and counted 4 times. Data were treated with computerized linear regression and rates of aminoacylation and AMP formation determined as average values from different countings and then from all experiments. The stoichiometry of AMP formation is given as the quotient of the rate of AMP formation and aminoacyl-tRNA formation. The total experimental error is estimated to be ±30%, including errors generated by AMP formation independent from the aminoacylation reaction which had been determined in control experiments without amino acid to be below 10%. From control experiments, it had been secured that K_m and k_{cat} values as well as AMP formation rates cannot be falsified by differences in substrate concentrations of the different assays applied in our work.

Results

Discrimination Factors in Aminoacylation of $tRNA^{Ile}$-C-C-A from k_{cat} and K_m Values. The discrimination factor *D* can be calculated from kinetic constants by the equation $D = (k_{cat}/K_m)_A(k_{cat}/K_m)_B^{-1}$. The k_{cat} and K_m values for aminoacylation of $tRNA^{Ile}$-C-C-A with isoleucine have been determined under different conditions in our previous work (Freist & Sternbach, 1984).

The reaction of $tRNA^{Ile}$ with valine and isoleucyl-tRNA synthetase from *Escherichia coli* was first studied by Hopfield et al. (1976) in the presence of the elongation factor Tu·GTP complex for protection of the aminoacylated tRNA from deacylation. Because a very slow but statistically highly significant misacylation rate could also be observed without addition of the elongation factor Tu·GTP complex, we carried out some experiments in the presence as well as in the absence of this compound. The findings of these studies are listed in Table I. The K_m value of valine is in the range 0.03–0.09 mM and thus appears, in nearly all cases, to be 2–3-fold higher than the K_m of the cognate substrate isoleucine. The k_{cat} values of about 0.0001 s^{-1} are, however, lower by a factor of 1000–10000 than for the cognate aminoacylation conducted under the same conditions. Whereas the quotient k_{cat}/K_m is relatively invariant in the misacylation reaction (Table I), it varies by up to a factor of 10 in the cognate aminoacylation mainly as a result of changes in k_{cat}. The highest discrimination ($D = 38\,000$) is observed in the standard assay optimized for best enzyme activity at pH 7.65. Lowering the pH to 6.5 causes a decrease in accuracy to $D = 8000$, whereas at a higher pH of 8.6 the accuracy decreases only to a *D* value of 14 000. When inorganic pyrophosphatase is added to the standard assay at pH 7.65, an insignificantly lower discrimination factor of 32 000 is observed. When the elongation factor Tu·GTP complex (EF-Tu·GTP) is present in the re-

Table I: Conditions, k_{cat} and K_m Values, and Resulting Discrimination Factors (D) in Aminoacylation of $tRNA^{Ile}$-C-C-A with Isoleucine [Taken from Freist & Sternbach (1984)] and Valine

conditions	Ile			Val			$D = (k_{cat}/K_m)_{Ile}/(k_{cat}/K_m)_{Val}$
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	
pH 6.5, 0.1 M KCl, 15 mM Mg^{2+}	0.41	0.03	13.7	0.0001	0.06	0.0017	8000
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}	0.83	0.02	41.5	0.0001	0.09	0.0011	38000
pH 8.6, 0.1 M KCl, 15 mM Mg^{2+}	0.52	0.02	26	0.00007	0.04	0.0018	14000
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, PPase	1.40	0.04	35	0.0001	0.09	0.0011	32000
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP	0.28	0.02	14	0.0001	0.06	0.0017	8000
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP, PPase	0.23	0.05	4.6	0.00009	0.05	0.0018	3000
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP, PPase, spermine	0.098	0.02	4.9	0.0001	0.05	0.0020	2000
pH 7.65, 0.1 M KCl, 1 mM Mg^{2+}, EF-Tu·GTP, PPase, spermine	0.135	0.008	16.9	0.00008	0.05	0.0016	10500
pH 9, 8 mM Mg^{2+}	0.01	0.02	0.5	0.0025	0.03	0.083	6

Table II: Conditions, k_{cat} and K_m Values, and Resulting Discrimination Factors (D_1) in Aminoacylation of $tRNA^{Ile}$-C-C-3′dA, $tRNA^{Ile}$-C-C-A(3′NH_2), and $tRNA^{Ile}$-C-C-2′dA with Isoleucine and Valine

conditions	Ile		Val		$D_1 = (k_{cat}/K_m)_{Ile}/(k_{cat}/K_m)_{Val}$
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	
$tRNA^{Ile}$-C-C-3′dA					
pH 6.5, 0.1 M KCl, 15 mM Mg^{2+}	0.215	0.0051	0.029	0.033	48
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}	0.20	0.002	0.005	0.009	180
pH 8.6, 0.1 M KCl, 15 mM Mg^{2+}	0.63	0.0088	0.016	0.021	94
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, PPase	0.28	0.002	0.028	0.02	100
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP	0.17	0.002	0.010	0.0085	72
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP, PPase	0.28	0.0018	0.036	0.016	69
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP, PPase, spermine	0.25	0.0017	0.045	0.026	85
pH 7.65, 0.1 M KCl, 1 mM Mg^{2+}, EF-Tu·GTP, PPase, spermine	0.21	0.0012	0.035	0.021	105
pH 9, 8 mM Mg^{2+}	0.055	0.003	0.0074	0.008	20
$tRNA^{Ile}$-C-C-A(3′NH_2)					
pH 6.5, 0.1 M KCl, 15 mM Mg^{2+}	0.093	0.005	0.0033	0.015	85
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}	0.10	0.006	0.002	0.02	167
pH 8.6, 0.1 M KCl, 15 mM Mg^{2+}	0.28	0.006	0.0086	0.02	109
$tRNA^{Ile}$-C-C-2′dA					
pH 9, 8 mM Mg^{2+}, PPase	0.001	0.009	0.0004	0.02	5.6

action mixture, discrimination is lowered to a value of D = 8000. Addition of pyrophosphatase to this mixture causes a further decrease in accuracy to D = 3000. Addition of spermine as a third additive has only a minor influence on the error rate. However, lowering the Mg^{2+} ion concentration to the physiological value of 1 mM in the presence of EF-Tu·GTP, pyrophosphatase, and spermine results in a higher discrimination factor of 10 500. As a last variation of assay conditions, the reaction mixture was adjusted to pH 9 at a very low salt concentration of 8 mM $MgSO_4$. Under these "misacylation conditions" (Giegé et al., 1972; 1974; Kern et al., 1972; Ebel et al., 1973), a very low discrimination factor of 6 was calculated.

Discrimination Factors in Aminoacylation of $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-A(3′NH_2) from k_{cat} and K_m Values. $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-A(3′NH_2) can be aminoacylated with isoleucine or with valine, exhibiting exactly linear aminoacylation kinetics which allow exact determinations of k_{cat} and K_m values (Freist & Cramer, 1983). This observation was explained by the assumption that lack of the terminal 3′-OH group at the tRNA causes a complete loss of the proofreading capacity of the enzyme (von der Haar & Cramer, 1976). From these k_{cat} and K_m values, as well as from those of the corresponding ATP/PP_i pyrophosphate exchange, a factor of about 180 was calculated for discrimination between isoleucine and valine (Cramer et al., 1981; Freist & Cramer, 1983; Fersht, 1977a,b, 1981; Fersht et al., 1980). This value was considered to result from a difference of about 3 kcal in the free enthalpy of binding in the transition state (Fersht, 1977a,b, 1981; Fersht et al., 1980).

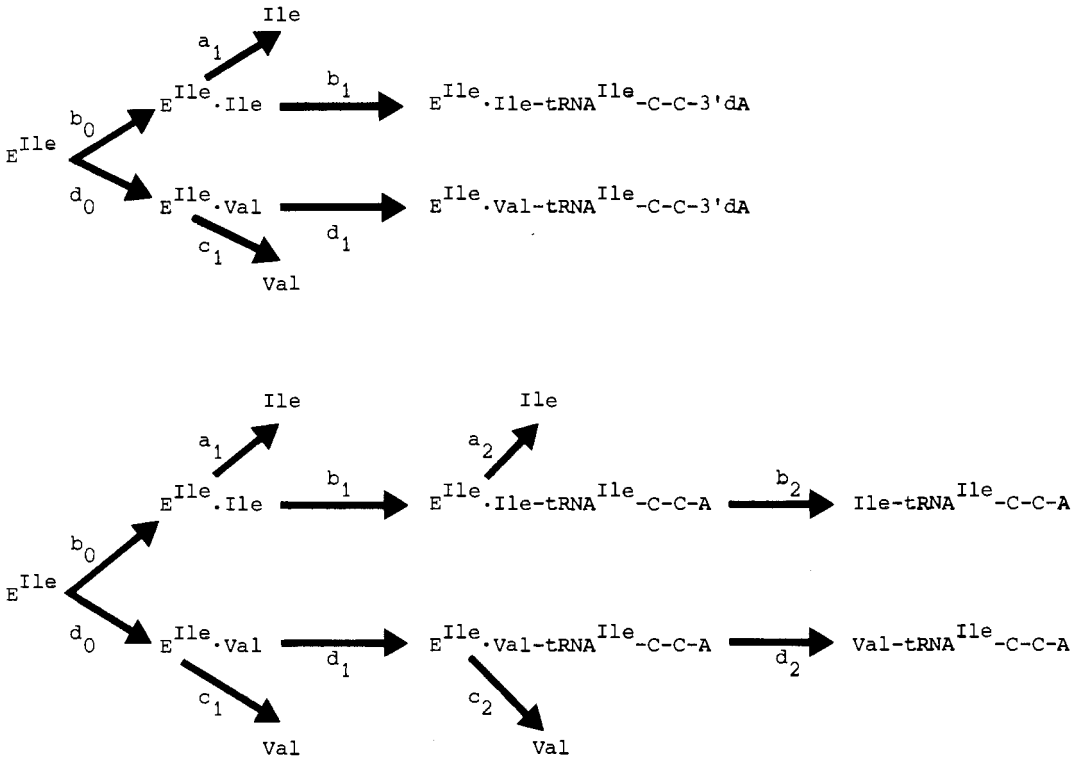
For calculation of error rates under the different conditions applied in our work, it seemed to be necessary to redetermine whether the factor 180 is constant or whether it is changed by different assay conditions. For this purpose, k_{cat} and K_m values of the aminoacylation of $tRNA^{Ile}$-C-C-3′dA with isoleucine and valine were determined by using the same conditions as for the aminoacylation of $tRNA^{Ile}$-C-C-A. The results which are listed in Table II show that the calculated discrimination factor in aminoacylation of $tRNA^{Ile}$-C-C-3′dA, which we call D_1, differs considerably. The maximum value of 180, which is obtained under the standard conditions at pH 7.65, is changed to a similar extent and with similar tendencies to those observed for the overall accuracy in the aminoacylation of $tRNA^{Ile}$-C-C-A (Table I).

It was shown that isoleucyl and valyl esters of $tRNA^{Ile}$-C-C-3′dA cannot be hydrolyzed at pH 7.6 by isoleucyl-tRNA synthetase (von der Haar & Cramer, 1976). In most of our experiments, the pH is also kept at 7.65, and furthermore, the EF-Tu·GTP complex is added to protect the aminoacylated tRNA and make hydrolysis even less likely. However, for acylation at pH 8.6 and 6.5, it had to be confirmed that Val-$tRNA^{Ile}$-C-C-3′dA is also stable under these conditions. Although no hydrolysis could be observed in experiments analogous to those of von der Haar & Cramer (1976), the experiments were also carried out with $tRNA^{Ile}$-C-C-A-(3′NH_2) which forms stable amide bonds with amino acids instead of potentially labile ester bonds (Table II). The results are in accordance with those obtained with $tRNA^{Ile}$-C-C-3′dA. They also strongly suggest that discrimination between isoleucine and valine in the ATP/PP_i pyrophosphate exchange

Table III: Conditions, Moles of AMP Formed per Mole of Aminoacyl-tRNA^{Ile}-C-C-3′dA, -tRNA^{Ile}-C-C-A(3′NH$_2$), and -tRNA^{Ile}-C-C-2′dA, Initial Discrimination Factors (I), Corresponding Difference in Free Binding Enthalpy (Calculated from $I = e^{-\Delta\Delta G/RT}$), and Proofreading Factors (P_1)

conditions	AMP per Ile-tRNA^{Ile}-C-C-3′dA ($=X_1$)	AMP per Val-tRNA^{Ile}-C-C-3′dA ($=Y_1$)	$I = D_1(X_1/Y_1)$	$\Delta\Delta G$ kcal	$\Delta\Delta G$ kJ	$P_1 = (Y_1 - 1)/(X_1 - 1)$
tRNA^{Ile}-C-C-3′dA						
pH 6.5, 0.1 M KCl, 15 mM Mg^{2+}	1.81	13	6.5	1.15	4.81	15
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}	1.94	96	3.6	0.79	3.31	101
pH 8.6, 0.1 M KCl, 15 mM Mg^{2+}	2.31	71	3.0	0.68	2.85	53
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, PPase	2.63	49	5.3	1.03	4.31	29
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP	1.25	19	4.7	0.95	3.98	72
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP, PPase	2.63	43	4.2	0.88	3.68	26
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu·GTP, PPase, spermine	3.12	48	5.5	1.05	4.39	22
pH 7.65, 0.1 M KCl, 1 mM Mg^{2+}, EF-Tu·GTP, PPase, spermine	1.1	20	5.7	1.07	4.48	190
pH 9, 8 mM Mg^{2+}	2.52	42	1.1	0.06	0.25	27
tRNA^{Ile}-C-C-A(3′NH$_2$)						
pH 6.5, 0.1 M KCl, 15 mM Mg^{2+}	3.5	97	3.0	0.68	2.85	39
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}	2.2	111	3.3	0.74	3.10	92
pH 8.6, 0.1 M KCl, 15 mM Mg^{2+}	3.0	106	3.1	0.70	2.92	53
tRNA^{Ile}-C-C-2′dA						
pH 9, 8 mM Mg^{2+}, PPase	250	1062	1.1	0.06	0.25	4.3

Scheme I: Mischarging Network of tRNA^{Ile}-C-C-3′dA and tRNA^{Ile}-C-C-A(3′NH$_2$) according to Savageau & Freter (1979) (Top) and Mischarging Network of tRNA^{Ile}-C-C-A according to Freter & Savageau (1980) (Bottom)



and in aminoacylation of modified tRNAs may be caused not only by differences in binding energies of the two substrates but also by a pretransfer correction step.

Determination of Initial Discrimination Factors from AMP Formation. To check whether the observed variations of the discrimination factor D_1 in aminoacylation of tRNA^{Ile}-C-C-3′dA with isoleucine and valine perhaps include a first hydrolytic correction step, the rate of AMP formation during aminoacylation with these two amino acids was measured. According to the reaction equation for aminoacylation of tRNA, for 1 mol of aminoacyl-tRNA 1 mol of AMP should be formed; consumption of more than one ATP molecule for one aminoacyl-tRNA in misacylation is indicative of a hydrolytic proofreading mechanism. When we measured the AMP production during aminoacylation of tRNA^{Ile}-C-C-3′dA with the cognate amino acid isoleucine, the quotient of AMP formation rate and isoleucylation rate (X_1) varied from 1.1 up to 3.12 mol of AMP/1 mol of Ile-tRNA^{Ile}-C-C-3′dA synthesized (Table III), indicating that this step indeed includes proofreading. Similarly, in aminoacylation of tRNA^{Ile}-C-C-3′dA and tRNA^{Ile}-C-C-A(3′NH$_2$) with valine for the corresponding quotient, Y_1 values between 13 and 111 were observed (Table III).

To calculate initial discrimination and proofreading factors of this pretransfer correction step, data were analyzed in a flux scheme introduced by Savageau & Freter (1979) (Scheme I). The upper branch of this network represents acylation with the cognate substrate isoleucine. AMP formation by hydrolysis of isoleucyl adenylate in a pretransfer proofreading step is given by flux a_1, and the portion of AMP which is produced by

acylation of $tRNA^{Ile}$-C-C-3′dA equals flux b_1. The quotient of AMP formation rate and acylation rate, X_1, is given by $X_1 = b_0/b_1 = (a_1 + b_1)/b_1$. Analogous relations are valid for the mischarging reaction: c_1 represents AMP formation by pretransfer proofreading, and d_1 is AMP production by acylation. The quotient Y_1 is given by $Y_1 = d_0/d_1 = (c_1 + d_1)/d_1$. If the enzyme recognizes the cognate substrate isoleucine better than noncognate valine by an initial discrimination factor I, the proportion of the upper branch of the scheme to the lower one will be $I = b_0/d_0 = (a_1 + b_1)/(c_1 + d_1)$. The discrimination factor D_1 is given by the ratio of fluxes of cognate and noncognate substrates and can be written as $D_1 = b_1/d_1 = IY_1/X_1$. Because D_1 has been determined from kinetic constants, the initial discrimination factor I can be calculated from the equation $I = D_1X_1/Y_1$. Savageau & Freter (1979) define the proofreading factor as the ratio of incorrect flux to that continuing on, c_1/d_1, divided by the ratio of correct flux to that continuing on, a_1/b_1. Thus, the proofreading factor P_1 is given by $P_1 = b_1c_1/a_1d_1 = (Y_1 - 1)/(X_1 - 1)$.

The calculated initial discrimination factors I are listed in Table III. They vary in the range from 3.0 to 6.5 under normal assay conditions, corresponding to a difference of 0.68–1.15 kcal in free binding enthalpy in the transition state. These values are numerically consistent with the factor calculated and measured by Pauling (1958) and Pauling & Pressman (1945). The proofreading factors P_1 are more variable and are in the range of 15–190.

$tRNA^{Ile}$-C-C-A(3′NH_2) (Table III) is aminoacylated with similar initial discrimination and proofreading factors as $tRNA^{Ile}$-C-C-3′dA, an observation which shows again that this factor does not depend on the modification type of the tRNA and is not falsified by hydrolysis of amino acid esters.

When aminoacylations of $tRNA^{Ile}$-C-C-3′dA or $tRNA^{Ile}$-C-C-2′dA with isoleucine and valine are carried out under the abnormal special "misacylation conditions" (pH 9, low salt concentration), initial discrimination factors of 1.1 are observed. These values confirm the assumption made in our previous work (Freist & Cramer, 1983) that, under these conditions, the amino acid is not bound in the normal binding pocket of the enzyme which would have allowed the discrimination of these amino acids by the binding energy of one methylene group.

Calculation of Discrimination Factors in Aminoacylation of $tRNA^{Ile}$-C-C-A from AMP Formation. In aminoacylation of $tRNA^{Ile}$-C-C-3′dA, AMP formation which must be due to a pretransfer proofreading step is observed. AMP production in aminoacylation of $tRNA^{Ile}$-C-C-A must also include a posttransfer step because the amino acid is transferred to the tRNA as can be seen by valylation of the modified tRNAs and because Val-$tRNA^{Ile}$-C-C-A is hydrolyzed by the enzyme (von der Haar & Cramer, 1976). From AMP production rates of both proofreading steps, overall discrimination factors D_{AMP} can be calculated which represent the discrimination between valine and isoleucine resulting from initial discrimination and two AMP-producing proofreading steps.

When $tRNA^{Ile}$-C-C-A is aminoacylated with isoleucine, X' moles of AMP is formed per one Ile-$tRNA^{Ile}$-C-C-A. This reaction is represented by the upper branch of Scheme I. AMP generated in the pretransfer proofreading step is given by flux a_1; AMP formation of the posttransfer step equals flux a_2; the part of AMP produced by aminoacylation of $tRNA^{Ile}$-C-C-A equals flux b_2. For X', the following equation is valid: $X' = (a_1 + a_2 + b_2)/b_2 = b_0/b_2$; $(b_0/b_1)(b_1/b_2) = X_1X_2$. Analogously in the flux scheme branch of Val-$tRNA^{Ile}$-C-C-A, the amount of AMP formed per one Val-$tRNA^{Ile}$-C-C-A is given by $Y' = (c_1 + c_2 + d_2)/d_2 = d_0/d_2 = (d_0/d_1)(d_1/d_2) = Y_1Y_2$. The overall discrimination factor D_{AMP} can be calculated as

$$D_{AMP} = \frac{b_2}{d_2} = \frac{b_0(b_2/b_1)(b_1/b_0)}{d_0(d_2/d_1)(d_1/d_0)} = I\frac{Y_1Y_2}{X_1X_2} = I\frac{Y'}{X'}$$

The proofreading factor P_2 is defined, analogously to P_1, as $P_2 = b_2c_2/a_2d_2 = (Y_2 - 1)/(X_2 - 1)$.

The measured AMP formation rates and calculated values for D_{AMP} are listed in Table IV. The last ones vary between 106 and 980, except for the very low value of 11 obtained under the special misacylation conditions (pH 9, 8 mM Mg^{2+}). These D_{AMP} values are significantly lower than the discrimination factors D obtained from kinetic measurements. The correction factor $\Pi = D/D_{AMP}$ varies between 15 and 70 (Table IV); only for the extreme conditions at pH 9, this factor is about 1. Strikingly, the values of Π are relatively constant (19–27) for all experiments carried out in the presence of EF-Tu·GTP complex. The difference between the discrimination factors D and D_{AMP} indicates that in aminoacylation of $tRNA^{Ile}$-C-C-A the enzyme acts with a different initial discrimination $I' = I\Pi$ or that an additional process takes place which enhances accuracy and does not result in AMP formation. It can be concluded that this step which might be responsible for correction factor Π must occur before the AMP-generating proof steps. Because per one Val-$tRNA^{Ile}$-C-C-A D molecules of Ile-$tRNA^{Ile}$-C-C-A are produced, more than $X'D$ molecules of AMP must be formed by isoleucylation, and the same number of substrate molecules must enter the upper branch of Scheme I. The number of substrate molecules which enter the lower branch representing valylation must be $\geq X'D/I$. If all these molecules would be subject to the first AMP-generating proofreading step, it would follow for the number of AMP molecules formed per one Val-$tRNA^{Ile}$-C-C-A $Y' \geq [(Y_1 - 1)/Y_1](X'D/I)$ or $Y_1/(Y_1 - 1) \geq X'D/IY' = D/D_{AMP} = \Pi$. Obviously, this is not observed under any of our reaction conditions (Table IV), and the non-AMP-generating correction step must diminish the numbers of substrate molecules which become proofread in the AMP-generating steps. Assuming such a model of proofreading, the number of AMP molecules formed per one aminoacyl-tRNA in the second proofreading step (X_2, Y_2) and proofreading factor P_2 can be calculated (Table IV). Only in one case (pH 6.5), P_2 is higher than P_1; for all other reaction conditions applied, P_1 shows higher values than P_2.

Discussion

For rejection of valine by isoleucyl-tRNA synthetase, differing models have been created (Hopfield et al., 1976; von der Haar & Cramer, 1976; Fersht, 1977b). In the "kinetic proofreading" mechanism, the same discrimination ability is used twice (Hopfield et al., 1976). The whole process results in the second power of the initial discrimination factor and was regarded as inconsistent with the experimental data obtained with isoleucyl-tRNA synthetase (Fersht, 1977b).

The "chemical proofreading" mechanism was based on the observations that modified tRNA species such as $tRNA^{Ile}$-C-C-A(3′NH_2) and $tRNA^{Ile}$-C-C-3′dA, in contrast to the natural substrate $tRNA^{Ile}$-C-C-A, can be very well aminoacylated with noncognate valine (von der Haar & Cramer, 1976). It was concluded that valine is first transferred to $tRNA^{Ile}$-C-C-A and then the valyl-tRNA ester bond is hydrolyzed before Val-$tRNA^{Ile}$-C-C-A can be released. To perform this hydrolysis, the 3′-hydroxyl group of the terminal ribose should be essential.

Fersht (1977b) discussed two possibilities for interpretation of his data. Because the measured rates of hydrolysis of

Table IV: Conditions, Moles of AMP Formed per Mole of aa-tRNA^{Ile}-C-C-A (X', Y'), Discrimination Factors (D_{AMP}) Calculated from AMP Formation, Correction Factors (II), Moles of AMP Formed per Mole of aa-tRNA^{Ile}-C-C-A in the Second Proofreading Step (X_2, Y_2), Proofreading Factors (P_2),[a] Quotient of Proofreading Factors, and Order of Substrate Addition in Aminoacylation with Isoleucine[b]

conditions	AMP per Ile-tRNA^{Ile}, X'	AMP per Val-tRNA^{Ile}, Y'	D_{AMP}	D (see Table I)	$\Pi = D/D_{AMP}$	$X_2 = X'/X_1$	$Y_2 = Y'/Y_1$	$P_2 = (Y_2 - 1)/(X_2 - 1)$	P_1/P_2	order of substrate addition and product release
pH 6.5, 0.1 M KCl, 15 mM Mg^{2+}	3.5	288	535	8000	15	1.93	22.2	23	0.7	sequential random Ter-Ter
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}	5.5	828	542	38000	70	2.84	8.6	4	25	sequential ordered Ter-Ter
pH 8.6, 0.1 M KCl, 15 mM Mg^{2+}	6.9	588	256	14000	55	2.99	8.3	4	13	sequential ordered Ter-Ter
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, PPase	6.3	1165	980	32000	33	2.40	23.8	16	1.8	random Bi-Uni Uni-Bi ping-pong
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu-GTP	3.0	192	301	8000	27	2.40	10.1	7	10	sequential ordered Ter-Ter
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu-GTP, PPase	5.1	165	136	3000	22	1.94	3.8	3	9	sequential ordered Ter-Ter
pH 7.65, 0.1 M KCl, 15 mM Mg^{2+}, EF-Tu-GTP, PPase, spermine	6.1	118	106	2000	19	1.96	2.5	2	11	Bi-Bi Uni-Uni ping pong
pH 7.65, 0.1 M KCl, 1 mM Mg^{2+}, EF-Tu-GTP, PPase, spermine	3.4	255	428	10500	25	3.09	12.8	6	32	Bi-Bi Uni-Uni ping-pong
pH 9, 8 mM Mg^{2+}	7.5	72	11	6	~1	2.98	1.7	~1	~27	sequential ordered Ter-Ter

[a] Calculated by assuming a preceding non-AMP-producing correction step. [b] A = ATP, B = isoleucine, C = tRNA^{Ile}-C-C-A, P = pyrophosphate, Q = Ile-tRNA^{Ile}-C-C-A, and R = AMP; taken from Freist & Sternbach (1984).

Val-tRNA^{Ile}-C-C-A were relatively low, he assumed that the enzyme may be in a hydrolytically more active conformation during the transfer of the aminoacyl residue, or that valyl adenylate may be hydrolyzed in a major editing step before the transfer, and that the hydrolytic activity of the enzyme toward Val-tRNA^{Ile}-C-C-A is only used to "mop up" any misacylated tRNA^{Ile} that leaks through the first editing step.

In previous works, misacylation of tRNA^{Ile}-C-C-3′dA and tRNA^{Ile}-C-C-A(3′NH$_2$) with valine (von der Haar, 1976; Freist & Cramer, 1983) has been regarded as a reaction without proofreading, and AMP formation in this step was considered as marginal (von der Haar & Cramer, 1976). Thus, discrimination factor D_1 had been regarded as to be caused only by the difference in binding energy of the two substrates isoleucine and valine. Indeed, if we calculate D_{AMP} using the values of D_1 for I in the equation $D_{AMP} = I(Y'/X')$, we get D_{AMP} values which are numerically of the same order of magnitude as the D values obtained by kinetic measurements. The deviation between D and D_{AMP} would be in the range of 30–50%, the difference in binding free enthalpy being 2.82–3.40 kcal (11.80–14.23 kJ). However, this simple analysis is no longer consistent with newer investigations. We interpret the results of our new measurements as a multistep proofreading model. This includes pretransfer and posttransfer proofreading steps which are both responsible for AMP formation. In this way, this analysis comprehends the pretransfer proofreading model created by Fersht (1977b) and the chemical proofreading mechanism (von der Haar & Cramer, 1976). The possibility of pre- and posttransfer proofreading was also found in experiments with amino acid analogues and isoleucyl-tRNA synthetase (Englisch, 1984) as well as for phenylalanyl-tRNA synthetase (Gabius et al., 1983). It should also be noted that Lin et al. (1984) subsequently postulated a pre- and posttransfer proofreading mechanism for phenylalanyl-tRNA synthetase and speculated that possibly also isoleucyl-tRNA synthetase could act in a similar way.

The different discrimination factors and AMP formation rates in acylation of tRNA^{Ile}-C-C-3′dA and tRNA^{Ile}-C-C-A-(3′NH$_2$) allowed us to compute the real initial discrimination factors I. Their values vary in the range from 3.0 to 6.5 and show a good coincidence with the "Pauling factor", which was measured (Pauling & Pressman, 1945) and calculated (Pauling & Pressman, 1945; Pauling, 1958) nearly 40 years ago.

The data are also consistent with factors which can be estimated from a hydrophobicity scale containing free energy changes for transfer of amino acid side chains from water to organic solvents (Nozaki & Tanford, 1971). The correlation between hydrophobicity of the amino acid side chains and factors I could be confirmed by determination of these factors for other amino acids (W. Freist and F. Cramer, unpublished results).

Hopfield & Yamane (1979) calculated the difference in binding energy between isoleucine and valine assuming 6–9 methyl equiv in the surrounding area of the critical methyl group. From consideration of molecular models and enzyme–substrate complexes elucidated by X-ray analysis [e.g., see Fersht (1977a) and Dickerson & Geis (1971)], a value of 2–4 methyl equiv would be more realistic, and this would result in an energy difference of 1–1.6 kcal/mol (4.2–6.7 kJ/mol). From these considerations, it no longer appears certain that an energy difference of ~3 kcal between binding of isoleucine and valine given in earlier works (Fersht, 1977a,b, 1981; Fersht et al., 1980; Freist & Cramer, 1983) is correct. We tend more to believe in the lower value of about 1 kcal found by Pauling (1958) and by ourselves in our present work.

The reason for the deviation of D_{AMP} from D by a factor II cannot finally be explained by our data. As pointed out above, an enzyme-catalyzed "mopping up" hydrolysis of acylated tRNAs can only play a marginal role. Apparently, between the initial discrimination (I) and the first AMP-producing proofreading (P_1), there exists a further selection step the molecular nature of which is yet unknown. One could discuss an energy relay phenomenon (Hopfield, 1980; Savageau, 1981) or an additional proofreading step which does not result in formation of AMP. Another possible explanation is based on kinetic reasons. The initial discrimination $I = b_0/d_0$ will be mainly caused by the difference in free binding enthalpy of the two substrates if the binding process approximates an equilibrium. In this case, I is approximatively given by the quotients of the binding constants of isoleucine and valine: $I \approx K_{Val}/K_{Ile} \approx \exp(-\Delta\Delta G/RT)$. If the steady-state population of enzyme–amino acid complexes is not similar to equilibrium conditions, factor I is mainly determined by the probability of transition into the following complexes of the reaction sequence. In aminoacylation of the modified tRNAs, equilibrium conditions may be approximated more closely for the binding step and factor I may be more closely related to the difference of free binding enthalpies.

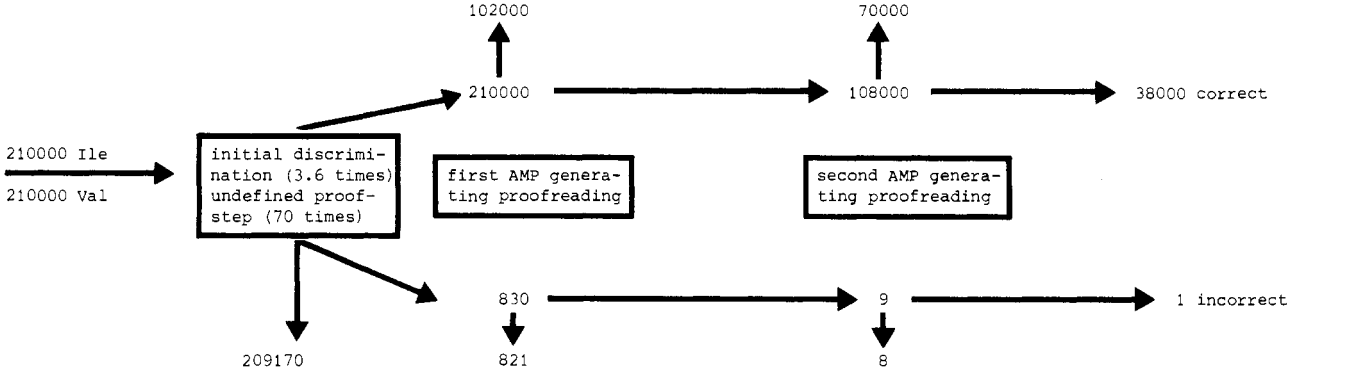
Because the initial discrimination factor has the low value of about 5, several correction steps are used to gain a sufficient specificity of the enzyme. These correction processes must be based on a principle other than the distinction by van der Waals forces of one methylene group. If the simple binding process would be used, e.g., 3 times, only a maximum discrimination of $I^3 = 75$ could be achieved. This excludes a mechanism similar to that proposed by Hopfield (1974) but favors a principle introduced by Fersht (1977a) and Fersht & Kaethner (1976b) for rejection of amino acids exhibiting smaller side chains than the cognate substrate. A smaller amino acid like valine is hydrolyzed from the tRNA in a hydrolytic site which is too small for an isoleucyl group to enter. Forcing isoleucine into this site would require a much higher energy than the difference in van der Waals binding forces of isoleucine and valine. Pauling (1958) measured a factor of about 100 by which methyl-substituted aromatic systems are bound less to antibodies. If it is assumed that the real "cramming factor" can be in the range of about 100, it is reasonable that more than one of such selection steps is necessary to achieve a suitable accuracy.

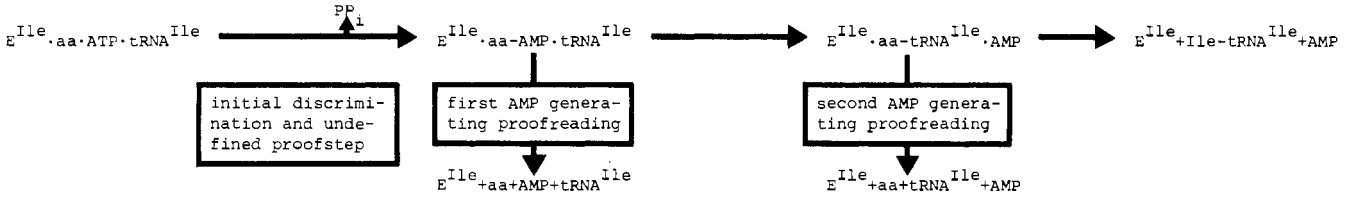
The first proofreading factor, P_1, varies in the range from 15 to 190 and exhibits an optimum value at pH 7.65 which is further increased in a reaction mixture containing only 1 mM Mg^{2+} besides EF-Tu·GTP complex, pyrophosphatase, and spermine (Table II). The pH optimum at 7.65 is in the same range as is observed for hydrolytically acting enzymes of the serine protease or carboxypeptidase type.

The second proofreading factor, P_2, varies between 1 and 23 and shows its highest value at pH 6.5, a pH optimum which is observed for enzymes of the thiol protease type. Only at this low pH is P_2 higher than P_1.

Consideration of the quotient P_1/P_2 obtained for different assay conditions gives a first hint as to the reason for the existence of distinctive aminoacylation pathways with different orders of substrate addition and product release (Table IV) (Freist & Sternbach, 1984). Strikingly, this quotient exhibits low values (0.7 and 1.8) for those aminoacylation pathways which show a random order in substrate addition. For sequential mechanisms, significantly higher values (9–32) are observed. The ability to act with different proofreading factors may enable the enzyme to adapt accuracy and proofreading

Scheme II: Initial Discrimination of Isoleucine and Valine by Isoleucyl-tRNA Synthetase and Multistep Proofreading upon Aminoacylation of $tRNA^{Ile}$ Quantified in Arbitrary Numbers of Molecules [(Top) Standard Assay Conditions (Compare Tables); (Bottom) Displayed as Formula Scheme] [a]





[a] From a pool containing isoleucine and valine in equal concentrations, isoleucine is $I' = 3.6 \times 70 = 252$ times more entering the AMP-forming proofreading processes than valine, e.g., 210 000 molecules of isoleucine in comparison to 210 000/252 = 830 molecules of valine. In the first step, $X_1 = 1.94$ molecules of AMP are generated per 1 molecule of isoleucine which is processed further. In this way, only 210 000/1.94 ≈ 108 000 isoleucine molecules can reach the second AMP-generating reaction. In the case of valine, $Y_1 = 96$ molecules of AMP are formed per 1 valine molecule which is further processed. Thus, 830/96 = valine molecules reach the next proofreading control. In the second step, per 1 isoleucyl-tRNA released finally from the enzyme 2.84 molecules of AMP are generated. The value of Ile-$tRNA^{Ile}$ synthesized is diminished to 108 000/2.84 = 38 000 molecules of isoleucyl-tRNA. Per 1 valine molecule which is finally released from the enzyme as valyl-tRNA, 8.6 molecules of AMP are formed in the second step. In this way, the value of nine molecules of valine which have reached the second proof step is reduced to about one.

costs to physiological requirements. Our results show clearly that the complete aminoacylation process, even with the cognate substrate isoleucine, requires a higher ATP consumption than postulated previously for the enzyme from *E. coli* (Fersht & Kaethner, 1976b; Hopfield et al., 1976) and that effective rejection of valine is only achieved by a considerable energy dissipation in an irreversible system [compare Prigogine & Lefever (1975)]. Consequently, this type of enzymatic process in which "chemical" flow and energy flow are intrinsically connected is strongly dependent on the details of the reaction conditions and can therefore easily be modulated. This might be typical for situations which are far away from any equilibrium. The maximum accuracy found in our experiments requires high amounts of ATP (Table IV). Under assay conditions which are similar to physiological ones (pH 7.65, 0.1 M KCl, 1 mM Mg^{2+}, pyrophosphatase, spermine), the enzyme acts with accuracy costs of about three ATPs for one correctly aminoacylated tRNA and achieves a medium value of accuracy of 1:10 500 (Table I) which may be just sufficient for cell function. It may very well be that isoleucyl-tRNA synthetase in yeast cells catalyzes the isoleucylation of $tRNA^{Ile}$ under conditions which allow a more economical mechanism having lower ATP consumption. A display of the fluxes of the substrates and products under standard assay conditions is given in Scheme II; under these conditions, 5.5 ATPs are consumed for formation of 1 isoleucyl-tRNA. It is reasonable to assume that other enzymes involved in the synthesis of biopolymers use the same principle of coupling molecular and energetical flow, thereby gaining both high accuracy and the capacity to modulate the cost/accuracy ratio.

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