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Molecular Recognition by Energy Dissipation, a New Enzymatic Principle: The Example Isoleucine-Valine[†]

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Introduction

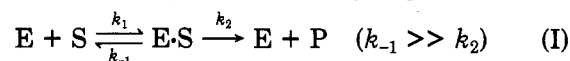
In the synthesis of biopolymers, the requirement for exact enzymatic discrimination between substrates of similar structure surpasses the capabilities of normal "lock and key" recognition. With some aminoacyl-tRNA synthetases (enzymes that select the individual amino acids for protein biosynthesis) specificity is achieved through a proofreading process which involves hydrolysis of additional ATP. This keeps the system far away from thermodynamic equilibrium. Whereas in most enzymatic reactions microreversibility is taken as a postulate, the multistep recognition of amino acids clearly occurs in an irreversible process that couples the molecular flow to an energy cascade.

Emil Fischer's Lock and Key Model

Some 90 years ago, E. Fischer discussed the substrate specificity of enzymes (Fermente) and, in particular, their capability to distinguish between enantiomers.¹ He came to the conclusion that the enzyme and substrate fit together like lock and key. Other similar molecules are excluded from the reaction by steric reasons. This ingenious paradigm arose at a time when

the nature of proteins was by no means clear, the concept of the macromolecule was not yet born, and the processes of life were still considered by most scientists to be outside the reach of scientific research.

The lock and key concept has proven to be extremely useful, and it can be considered a fundamental theorem of biochemical reactions. The Michaelis-Menten theory followed as a direct consequence.²⁻⁴ The enzyme "pocket" (or lock) is reversibly occupied and checked by the substrate (key) in a non-rate-determining step which is followed by a slower catalytic process.



The formation of the enzyme-substrate complex occurs approximately at thermodynamic equilibrium; the ΔG value of the overall process is solely determined by the chemical transformation of S into P. The specificity of the enzymatic reaction is determined by the ratio of the equilibrium constants for formation of the correct enzyme-substrate complex vs. the formation of the incorrect complex. This quotient can be regarded as the "initial discrimination". In principle, there could also be a difference between the "correct" and "incorrect" substrates in the catalytic step (k_2 in eq 1). In this case, the transition state of the reaction is more favorable for the correct substrate.³

The geometric details of the lock and key model were confirmed in the 1960s, when the three-dimensional structures of proteins became available.^{5,6} The theory

Friedrich Cramer was born in 1923 in Breslau. He studied chemistry at the Universities of Breslau (now Wrocław) and Heidelberg and completed his doctoral studies at the Institute of Karl Freudenberg in 1949. In 1953/1954 he spent a postdoctoral year at the Institute of A.R. Todd in Cambridge. In 1959 he was appointed professor at the Technical University of Darmstadt and in 1962 Head of the Chemistry Department of the Max-Planck-Institute for Experimental Medicine in Göttingen. He has investigated details of the enzymatic mechanisms and accuracy of protein biosynthesis, the three-dimensional structure of nucleic acids, and methods for synthesis of ribo- and deoxyribopolynucleotides. He is also author of two books on the responsibility of scientists in the modern world.

Wolfgang Freist was born in Bremen, Germany. He studied chemistry at the University of Kiel and received his Ph.D. in 1968. He did postdoctoral work on oligonucleotide synthesis with Prof. Dr. F. Cramer at the Max-Planck-Institute for Experimental Medicine in Göttingen. He is currently investigating the accuracy of aminoacyl-tRNA synthetases.

[†]Dedicated to Linus Pauling on the occasion of his 85th birthday.
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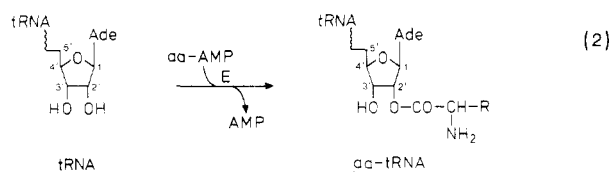
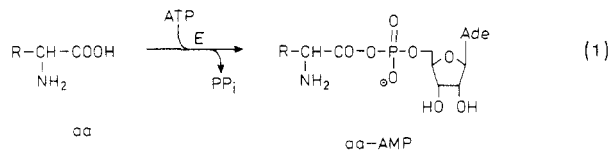
was also supported by model studies with the cyclodextrins. These molecules have a defined hollow space which allows molecular recognition and even enzymelike catalysis.⁷⁻⁹

Linus Pauling's Dilemma

Some 30 years ago, Linus Pauling initiated a lively discussion on the problem of fitting molecules one into the other and on the problem of how precisely enzymes can work in principle. Pauling had studied the specificity of complex formation between antibodies and their corresponding haptens by slight variation of the structure of the hapten. He came to the conclusion that structures varying, for instance, by one methylene group (e.g., methyl vs. ethyl) would differ in the specificity of binding by only a factor of 2-10.^{10,11} This would have grave consequences for the biosynthesis of proteins (and other biopolymers). A protein containing some 100 amino acids could never be synthesized as a homogeneous species if the error rate for incorporation of an amino acid was in the order of several percent. Pauling came to the disastrous conclusion "that the gene-controlled process of incorporation of alanine in its proper place in a polypeptide chain could not operate to exclude glycine completely; and that, indeed, errors in the alanine position should occur to an extent estimated at 5 percent. The same argument permits the same error rates for insertion of valine instead of isoleucine." It is self-evident that this cannot be the true situation in protein biosynthesis. There existed an unresolved dilemma.

The Aminoacylation of tRNA

The individual amino acids are selected from the cytoplasmic pool by the aminoacyl-tRNA synthetases. These enzymes fix the selected amino acids to the terminal ribose moiety of the corresponding transfer ribonucleic acid (tRNA) in a two-step reaction: the activation step (1) which requires adenosine triphosphate (ATP) to produce an aminoacyl adenylate (aa-AMP) and the transfer step (2) during which the amino acid becomes attached to the tRNA. The product of the activation step, the aa-AMP, remains enzyme bound.



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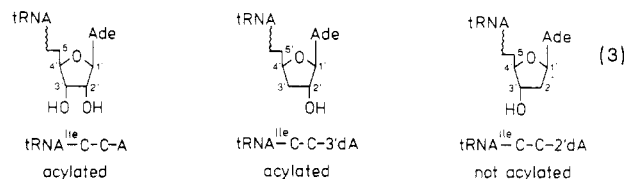
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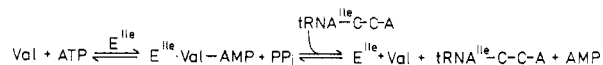
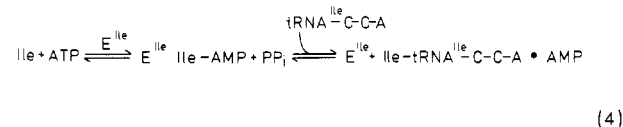
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In principle, the amino acid can be fixed to either the 2'- or the 3'-position of the terminal ribose¹²⁻¹⁶ of the tRNA. In the case of isoleucine (Ile; R = -CH(CH₃)-CH₂-CH₃) with which we shall deal further, the amino acid is attached to the 2'-OH group. This was determined by using modified tRNAs, lacking either the 2'- or the 3'-OH group,¹⁷⁻¹⁹ in the aminoacylation reaction (3). Besides the natural tRNA specific for isoleucine,



tRNA^{Ile}(tRNA^{Ile}-C-C-A), only the modified species lacking the 3'-OH group (tRNA^{Ile}-C-C-3'dA) was aminoacylated by the enzyme. This indicates that the 2'-OH group is essential for this reaction and that this must be the position to which isoleucine is bound. A first hint for the solution of Pauling's dilemma came when P. Berg and co-workers found that valine (Val; R = -CH(CH₃)-CH₃) is also activated by the isoleucyl-tRNA synthetase but, in contrast to isoleucine, is not transferred to tRNA^{Ile}.^{20,21} Addition of tRNA^{Ile} to the enzyme-valyl adenylate complex (E^{Ile}.Val-AMP) causes hydrolysis to valine and AMP (4) (AMP = adenosine monophosphate, PP_i = pyrophosphate, E^{Ile} = isoleucyl-tRNA synthetase). Apparently, there op-



erates a hydrolytic proofreading mechanism which rejects the misactivated amino acid before it is transferred to the tRNA. However, it was later demonstrated that enzyme-bound misaminoacylated tRNA (Val-tRNA^{Ile}-C-C-A) does form in the reaction but is hydrolyzed by the aminoacyl-tRNA synthetase before being released.^{22,23}

When Berg's experiment was repeated with tRNA^{Ile}-C-C-3'dA, instead of tRNA^{Ile}-C-C-A, this tRNA was completely misaminoacylated with valine^{24,25} (5). Since the only chemical difference between tRNA^{Ile} and tRNA^{Ile}-C-C-3'dA is the absence of the nonaccepting 3'-OH group of the terminal ribose, the 3'-OH group must be instrumental in the proofreading hydrolytic

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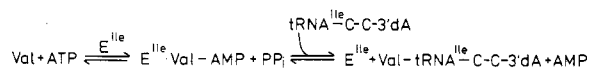
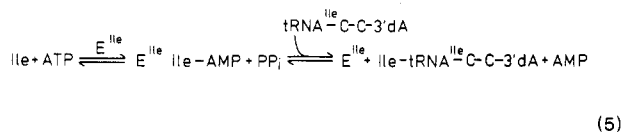
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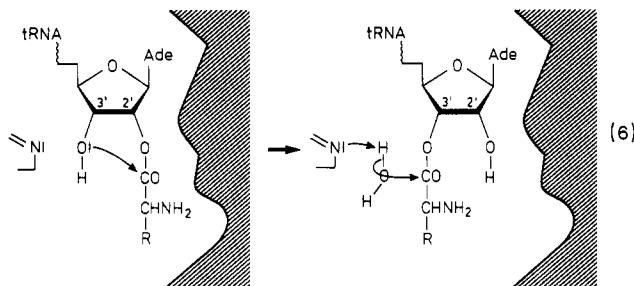


process. Therefore, the two terminal OH groups serve opposite purposes. The 2'-OH is the accepting site, and the 3'-OH is functional in editing.

Chemical Proofreading

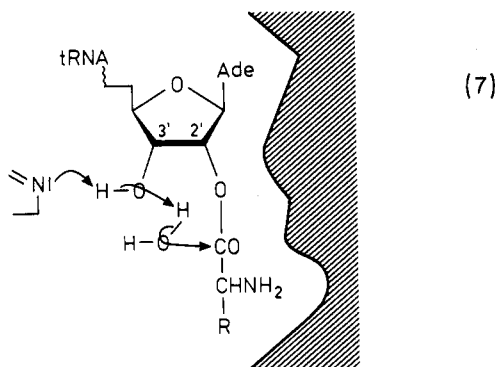
The selection and subsequent attachment of the amino acid to the tRNA is carried out by the same enzyme that also catalyzes the proofreading hydrolysis. The two processes must, however, not necessarily occur in the same enzymatic site. We have proposed two alternative mechanisms.

In the first mechanism, we propose that the amino acid migrates by transacylation to the 3'-OH and is subsequently removed from this position by a hydrolytic process similar to that operating in serine proteases²⁶ (6). In the second mechanism hydrolysis occurs



without migration of the aminoacyl residue. This hydrolysis is mediated by the nonaccepting hydroxyl group which fixes the hydrolyzing water molecule.^{24,25,27}

Any misfit between enzyme and amino acid will favor the insertion of a water molecule into the cavity of the enzyme (7).



It has also been proposed that the system could operate by a mechanism that sorts the amino acids by the size of two different active site cavities. Molecules larger than the substrate are excluded because they do not fit the active site; however, smaller molecules can bind and act as substrates. These smaller substrates are rejected by the second site. This special hydrolytic site has a binding cavity smaller than the cognate

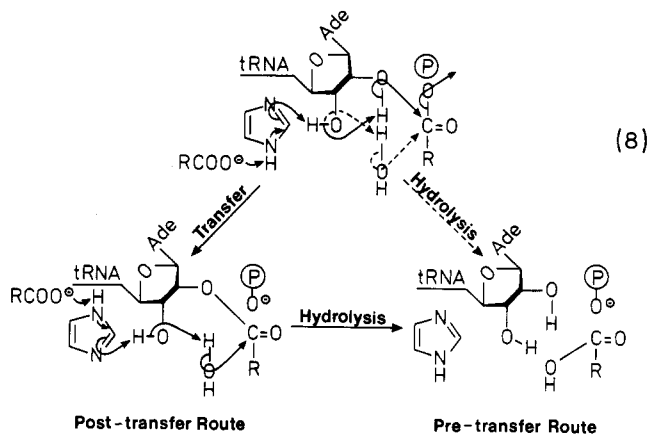
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substrate, and only the smaller substrates suffer hydrolysis.^{28,29}

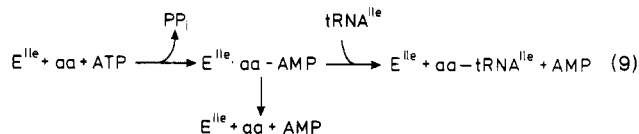
As it turned out later, the proofreading process does not necessarily have to occur by hydrolysis of the misaminoacylated tRNA but can also be achieved by hydrolysis of the aminoacyl adenylate prior to its transfer of the amino acid to the tRNA. The extent of pre-transfer proofreading and posttransfer proofreading can vary in different systems. From a systematic study with phenylalanyl-tRNA synthetases from several organisms the pretransfer route seems to be preferred by higher organisms.³⁰⁻³²

The chemical similarity of pre- and posttransfer proofreading processes is shown by the following hypothetical scheme:



First Quantitative Estimations

In one of the first theoretical considerations on proofreading phenomena, J. J. Hopfield, in 1974, proposed kinetic proofreading^{33,34} as a mechanism for reducing errors in biosynthetic processes requiring high specificity. It was predicted that a fine tune selection can be achieved after formation of the Michaelis-Menten complex if in a subsequent step the reaction is driven, by an energy-consuming process, irreversibly through a branching point in which editing can occur. In the aminoacylation of tRNAs, formation of aminoacyl adenylates is considered an irreversible ATP-consuming process, and the reaction sequence is branched by the possibility of adenylate hydrolysis. The noncognate substrates are preferentially rejected at this step (9).



The scheme Hopfield describes is valid for any proofreading process consisting of the combination of a specific coupling process followed by a specific hydrolytic process. If these two selection steps are physically independent processes, their specificities multiply.

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(32) Rauhut, R.; Gabius, H.-J.; Engelhardt, R.; Cramer, F. *J. Biol. Chem.* 1985, 260, 182.

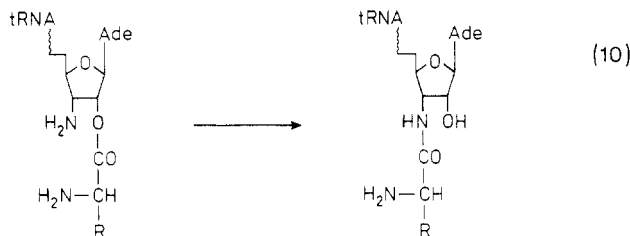
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For discrimination between isoleucine and valine by isoleucyl-tRNA synthetase from *E. coli* Hopfield made an estimation of the overall specificity resulting from the "Michaelis" step and the proofreading step. For the first, a discrimination rate of 1:100 was calculated from kinetic constants of the isoleucyl and valyl adenylate formation reactions.³⁴ The contribution of the proofreading step was determined from the rates of AMP formation. In synthesis of 1 molecule of Ile-tRNA^{Ile} 1.5 molecules of AMP were generated, whereas formation of Val-tRNA^{Ile} resulted in generation of 270 AMP molecules. From these values a proofreading discrimination of $270/1.5 = 180$ was calculated. Thus, the overall discrimination was estimated as $(1:100) \times (1:180) \approx 1:18\,000$.^{33,34}

Quantitative Measurements with tRNA^{Ile}-C-C-A(3'NH₂)

More detailed studies on these discrimination processes became feasible using a modified tRNA in which the terminal 3'-OH group was substituted by an amino group.¹⁷⁻¹⁹ If this tRNA^{Ile}-C-C-A(3'NH₂) is aminoacylated, stable amide bonds are formed by intramolecular aminolysis instead of the normal ester bonds (10). Thus, proofreading events observed in amino-



acylation of this tRNA can only occur before transfer of the amino acid to the tRNA and not by hydrolysis of the acylated tRNA. As an example of a detailed analysis of proofreading processes, the discrimination between isoleucine and valine by isoleucyl-tRNA synthetase from yeast will be described.

tRNA^{Ile}-C-C-A(3'NH₂) is easily aminoacylated with isoleucine or valine.³⁵ The specificity, given as the quotient for the initial velocities of acylation of the two substrates, can be written

$$v_{\text{Ile}}/v_{\text{Val}} = D([\text{Ile}]/[\text{Val}]) \quad (\text{II})$$

The discrimination factor D can be calculated from kinetic constants:

$$D = (k_{\text{cat}}/K_{\text{M}})^{\text{Ile}} / (k_{\text{cat}}/K_{\text{M}})^{\text{Val}} \quad (\text{III})$$

Under optimal assay conditions for acylation of tRNA^{Ile}-C-C-A(3'NH₂), with isoleucine and valine a value of

$$D_1 = (0.10 \text{ s}^{-1}/0.006 \text{ mM}) / (0.002 \text{ s}^{-1}/0.02 \text{ mM}) = 167$$

was determined. This means that in presence of equal amounts of isoleucine and valine the cognate substrate is incorporated 167 times more often than valine.³⁵

In acylation of tRNA^{Ile}-C-C-A(3'NH₂) with isoleucine 2.2 molecules of AMP are generated per one aminoacyl-tRNA³⁶ (X_1). The number of AMP molecules formed per one Val-tRNA^{Ile}-C-C-A(3'NH₂) is 111 (Y_1). The ratio $Y_1/X_1 = \pi_1 = 50$ indicates how many times

more valine is rejected in the pretransfer proofreading step than isoleucine. From the overall discrimination factor D_1 and π_1 the factor caused by the "Michaelis step", the initial discrimination factor J_1 , can be calculated as $J_1 = D_1/\pi_1 = 3.3$, and indeed this value is consistent with the factor estimated by Pauling 30 years ago.

Quantitative Measurements with tRNA^{Ile}-C-C-A

More complicated selection processes were observed for discrimination between isoleucine and valine in aminoacylation of tRNA^{Ile}-C-C-A, the naturally occurring tRNA species.³⁶ From kinetic constants the overall discrimination factor D (eq III) could be estimated as

$$D = (0.83 \text{ s}^{-1}/0.02 \text{ mM}) / (0.001 \text{ s}^{-1}/0.09 \text{ mM}) = 38\,000$$

In aminoacylation of tRNA^{Ile}-C-C-A, 5.5 mol of AMP was formed per mole of aminoacyl-tRNA (X) (a value which is remarkably higher than that one of 1.1 AMP observed with isoleucyl-tRNA synthetase from *E. coli* (Freist, W.; Cramer, F., in preparation)). Aminoacylation with valine resulted in formation of 828 mol of AMP (Y). The considerably higher ATP consumption, when compared to the results with tRNA^{Ile}-C-C-A(3'NH₂), indicates that a second posttransfer proofreading step exists. This proofreading process contributes a factor of ($\pi' = 828/5.5$) 150 (Scheme I) by which valine is rejected more often than isoleucine. Assuming that the contribution of the pretransfer proofreading step in aminoacylation of tRNA^{Ile}-C-C-A is as high as in aminoacylation of tRNA^{Ile}-C-C-A(3'NH₂), a proofreading factor (π_2) for the posttransfer step of 3 ($\pi_2 = \pi'/\pi_1 = 151/50$) can be calculated. The magnitude of this value shows that under optimal assay conditions the posttransfer hydrolytic correction is much less effective than the pretransfer hydrolysis. A similar result was obtained for isoleucyl-tRNA synthetase from *E. coli*.^{37,38} This factor for posttransfer proofreading may again be assigned to the difference in free binding enthalpy caused by lack of one methylene group in the valine molecule.

The initial discrimination factor (J'), computed as ($J' = D/\pi' = 38\,000/151$) 252, is considerably higher by a factor of ($J_2 = J'/J_1 = 252/3.3$) 76 than in aminoacylation of tRNA^{Ile}-C-C-A(3'NH₂). The values of (J_1) 3.3 and (J_2) 76 correspond to differences in free binding enthalpies of 3.08 (0.74) and 11.17 kJ/mol (2.67 kcal/mol) if calculated as $\Delta\Delta G_{J_1} = RT \ln J_1$ and $\Delta\Delta G_{J_2} = RT \ln J_2$. Whereas the first value is consistent with the contribution of a methylene group in a hydrophobic interaction, the second value cannot be easily explained; in our opinion the higher energy value must be due to a conformational change of the enzyme when binding the wrong substrate, which can only take place if the substrate does not fit the total binding site like a key its lock. In aminoacylation reactions of tRNA^{Ile}-C-C-A(3'NH₂) these processes may be blocked, an assumption which is supported by different kinetics in complex formation of aminoacyl-tRNA synthetases and tRNA^{Ile}-C-C-A and tRNA^{Ile}-C-C-3'dA.³⁹ Calculations of the hydrophobic forces of the binding of the substrate

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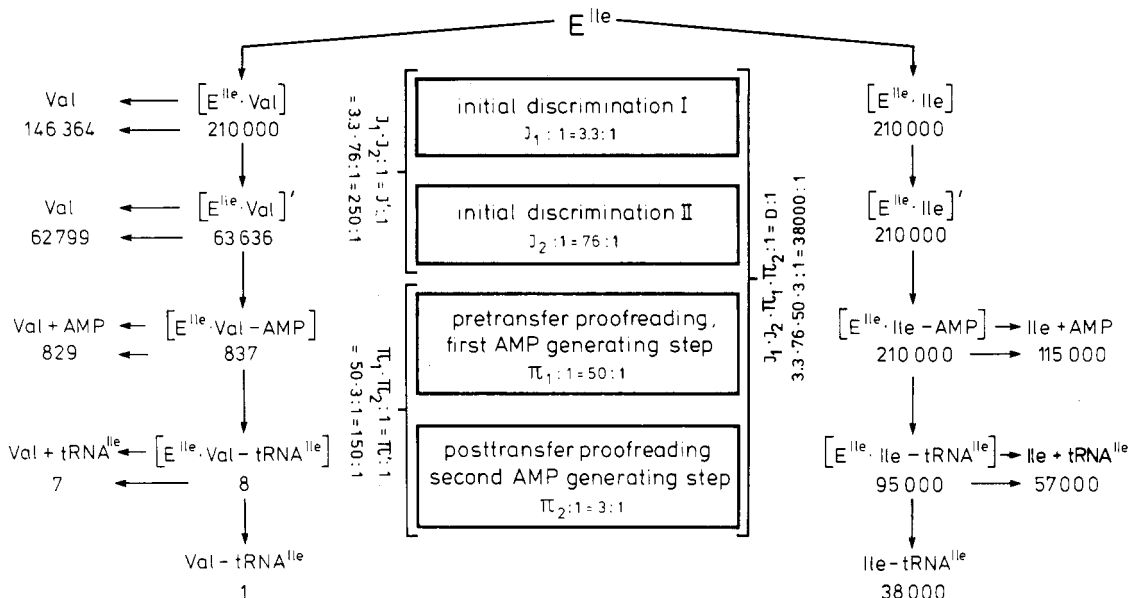
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Scheme I.
Discrimination Tree for Isoleucine and Valine by Isoleucyl-tRNA Synthetase Including Two Reversible Selection Processes and Two Irreversible Branching Points

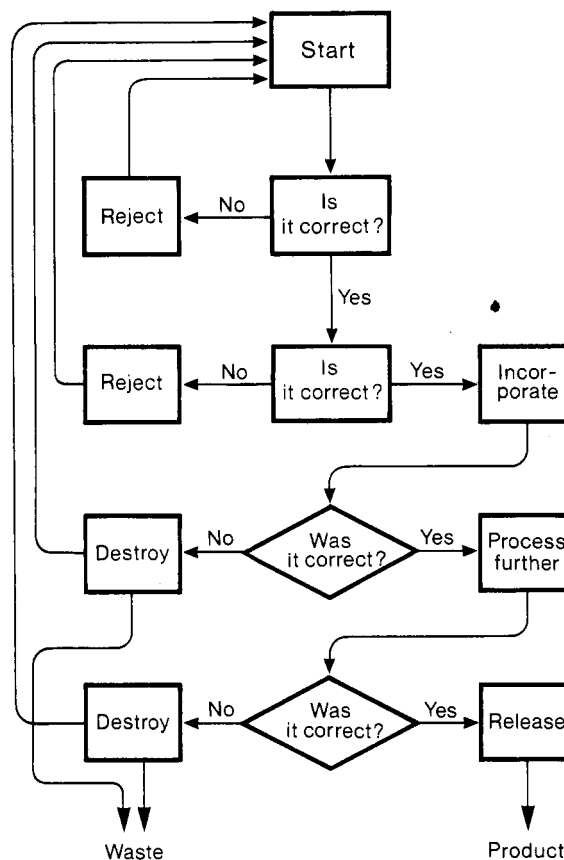


to the enzyme together with a schematic model of the active site of the enzyme may offer some theoretical insight in the problem (Freist, W.; Sternbach, H.; Cramer, F., in preparation).

The flux of substrate to product that occurs in the discrimination between isoleucine and valine by isoleucyl-tRNA synthetase from yeast is displayed as a "discrimination tree" in Scheme I. From a pool containing isoleucine and valine in equal concentrations, valine is [after the "initial discrimination step I" ($J_1 = 3.3$) and the "initial discrimination step II" ($J_2 = 76$)] 251 times less likely to enter the following AMP-forming process than isoleucine. For example, whereas the initial 210 000 molecules of isoleucine will survive these steps there will only be $(210\,000/3.3 \cdot 76 = 210\,000/251)$ 837 molecules of valine at this stage. In the first proofreading step, 2.2 molecules of AMP (X_2) are generated per molecule of isoleucine which is processed further. In this way only $(210\,000/2.2)$ 95 000 isoleucine molecules can reach the second AMP-generating reaction. In the case of valine (Y_1) 111 molecules of AMP are formed per valine molecule which is processed further. Thus, $(864/111)$ 8 valine molecules reach the next proofreading control. In the second step 2.5 molecules of AMP are generated per molecule of Ile-tRNA^{Ile} finally released from the enzyme. The value of Ile-tRNA^{Ile} is diminished to $(95\,000/2.5)$ 38 000 molecules of Ile-tRNA^{Ile}. For every valine molecule that is finally released from the enzyme as Val-tRNA^{Ile} in the second step 7.5 molecules of AMP are formed. In this way the value of about 8 molecules of valine which have reached the second proof step is reduced to 1.

This new type of enzymatic reaction is essentially an intimate coupling between a molecular flow of amino acids and an energy flow through ATP hydrolysis. It is therefore a driven reaction, operating far away from thermodynamic equilibrium. This is displayed as a switch diagram in Scheme II. The enzyme selects from the amino acid pool the cognate substrate, asking first "Is the binding amino acid the correct one?" If the answer is "no", the amino acid is rejected. If the answer is "yes", the amino acid will be considered further. This

Scheme II



reversible process may be repeated. Finally, a product is formed in a chemical reaction. Mistakes in these initial selection steps are eliminated in the third step, in which the enzyme asks "Was the selection correct?" If the answer is positive, the amino acid reaches the fourth step; in the negative case the acid is rejected. If the fourth step results also in a "yes" answer, the product is released; if the answer is "no", it will be hydrolyzed.

A process far away from thermodynamic equilibrium, dissipating energy, can be tuned much finer than a

Table I.
Reaction Conditions, Discrimination Factor D ,
Moles of AMP per Mole of Ile-tRNA^{Ile}, X' , and per Mole of
Val-tRNA^{Val}, Y' (0.1 M KCl, 15 mM Mg²⁺
Unless Otherwise Stated)

conditions	D	X'	Y'
pH 6.5	8 000	3.5	288
pH 7.65	38 000	5.5	828
pH 8.6	14 000	6.9	588
pH 7.65, PPase	32 000	6.3	1165
pH 7.65, EF-Tu-GTP	8 000	3.3	192
pH 7.65, EF-Tu-GTP, PPase	3 000	5.1	165
pH 7.65, EF-Tu-GTP, PPase, spermine	2 000	6.1	118
pH 7.65, 1 mM Mg ²⁺ , EF-Tu-GTP, PPase, spermine	10 500	3.4	255

process near equilibrium.⁴⁰ Therefore, by slight variation of parameters the accuracy can be lowered, and at the same time the ATP consumption is also lower. Examples for variation of accuracy and ATP consumption are given in Table I. Under conditions of the standard assay (pH 7.65) the enzyme exhibits its highest accuracy ($D = 38\,000$) and high ATP consumption. Lowering the pH to 6.5 or increasing it to 8.6 results in lower accuracies and lower energy dissipation. In the presence of inorganic pyrophosphatase (PPase) the enzyme acts with high specificity and the highest rates of AMP generation. When the elongation factor Tu-GTP complex (EF-Tu-GTP) is present in the reaction mixture, discrimination and ATP consumption are considerably lowered. Addition of pyrophosphatase to this mixture causes further decrease of accuracy to $D = 3\,000$ at similar AMP generation. Addition of spermine as a third additive has only minor influence on accuracy and ATP consumption. However, lowering the Mg²⁺ concentration to 1 mM in the presence of EF-Tu-GTP, pyrophosphatase, and spermine results in a medium value of factor D and ATP consumption. This last example may represent the best approximation to physiological conditions.

The discrimination isoleucine-valine varies by a factor of about 20, and ATP consumption varies by a factor of 2 in the cognate aminoacylation and by a factor of 10 in the noncognate reaction. Therefore, energy-consuming enzymes as isoleucyl-tRNA synthetase may function as a regulatory unit within an organism.

Concluding Remarks

In this review we have only discussed one step of protein biosynthesis. The entire assembly process of polypeptides, under the control of genes, requires at least two more selection steps: the synthesis of messenger RNA by RNA polymerase and the ribosomal codon/anticodon checking. Nothing is known, so far, on proofreading processes in the synthesis of messenger RNA. In the ribosomal process there also seems to be a proofreading mechanism operating.⁴¹⁻⁴⁶ However, the enzymatic system of the ribosomes is much more com-

plex and therefore it is difficult to study such mechanisms in the same detail as we can with the aminoacylation of tRNAs by the appropriate amino acids.

The overall error in protein biosynthesis will be determined by the least accurate step. Thus, evolution should have worked toward an equalization of the accuracy of the various steps as a compromise between functionality of the protein and economy of the system. Therefore, we would like to predict that the error in all steps of gene expression is of the same order as we have found here.

In the present concepts of mechanisms of enzymes according to the Michaelis-Menten theory and its modern extensions, selections and transitions occur near equilibrium. This is not the case in the class of biochemical reactions discussed in this article. In these reactions there are selection steps which operate according to simple mass law, but in addition there are steps which are driven by energy dissipation.^{33,34,47} The coupling of energy flow and molecular flow drives the system far away from equilibrium and renders it much more sensitive. According to Prigogine, at equilibrium matter is dull; the further one goes away from equilibrium, the more intelligent and sensitive matter becomes.⁴⁸

Alternatively, the system may be regarded as a nonlinear selection system with branching points in the course of the reaction. The decisions found at these branching points then are mandatory for the next alternatives of the reaction course. Such systems can be treated in the terms of synergetics. Other systems, which have been treated in this way, are the immune system,⁴⁹ the branched system of evolution,⁵⁰ or the pattern formation in biological systems.⁵¹ In our case, the selection tree (Scheme I) is a branched system, for creating a higher degree of order among the amino acids, and it is driven by energy consumption. Thus, an enzymatic protein can distinguish at equilibrium between isoleucine and valine only with an accuracy of 1 in 20, but when dissipating energy, 1 in 38 000. Moreover, if we define intelligence as the capacity to analyze a situation and to react accordingly, enzymes become "intelligent" if they operate far away from equilibrium. For instance, an organism could, in time of starvation, save energy (ATP) and synthesize its protein with less fidelity.

Clearly, life is a nonlinear situation far away from thermodynamic equilibrium. Therefore, we should not be surprised to find that a simple partial process of life like the aminoacylation of tRNA operates on a high-energy level through the dissipation of energy.

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Registry No. Isoleucyl-tRNA synthetase, 9030-96-0; isoleucine, 73-32-5; valine, 72-18-4; aminoacyl-tRNA synthetase, 9028-02-8.

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