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Mechanisms of Aminoacyl-tRNA Synthetases: A Critical Consideration of Recent Results

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ABSTRACT: During the last 10 years intensive and detailed studies on mechanisms and specificities of aminoacyl-tRNA synthetases have been carried out. Physical measurements, chemical modification of substrates, site-directed mutagenesis, and determination of kinetic parameters in misacylation reactions with noncognate amino acids have provided extensive knowledge which is now considered critically for its consistency. A common picture emerges: (1) The enzymes work with different catalytic cycles, kinetic constants, and specificities under different assay conditions. (2) Chemical modifications of substrates can have comparable influence on catalysis as can changes in assay conditions. (3) All enzymes show a specificity for the 2′- or 3′-position of the tRNA. (4) Hydrolytic proofreading is achieved in a pre- and a posttransfer process. In most cases pretransfer proofreading is the main step; posttransfer proofreading is often marginal. (5) Initial discrimination of substrates takes place in a two-step binding process. For some investigated enzymes, initial discrimination factors were found to depend on hydrophobic interaction and hydrogen bonds. (6) The overall recognition of amino acids is achieved in a process of at least four steps. At present, only a rough overall picture of aminoacyl-tRNA synthetase action can be given.

Soon after the first aminoacyl-tRNA synthetases were discovered, a general reaction mechanism was postulated by which these enzymes catalyze aminoacylation of tRNAs [for compilations, see Loftfield (1972), Kisselev and Favorova (1974), and Söll and Schimmel (1974)]. According to this mechanism, which is now generally accepted, amino acids are first converted to aminoacyladenylates in the activation step and then attached to tRNA in the transfer step.

$$E + aa + ATP \rightarrow E{\cdot}aa\text{-}AMP + PP_i$$

$$E{\cdot}aa\text{-}AMP + tRNA \rightarrow E + aa\text{-}tRNA + AMP$$

In contrast to this general opinion, some workers have insisted on a one-step mechanism (Loftfield, 1972; Loftfield & Eigner, 1969; Lövgren et al., 1975; Deutscher, 1967; Parfait et al., 1972; Thiebe, 1983), and it was also assumed that other unknown reactive intermediates formed with amino acids may play a role in aminoacylation (Thiebe, 1975; Kovaleva et al., 1983).

In spite of the fact that not all questions concerning the chemical mechanism had been solved, the main interest of most research groups turned to the problem of amino acid specificity of the enzymes. Investigations on the reasons for the high enzyme accuracies were carried out with physical methods such as rapid kinetic experiments, equilibrium dialysis, and filtration as well as chemical methods such as modification of substrates, and tests of reactivity of these compounds were applied. Sometimes results obtained by these different methods seemed to be in contradiction and were the subject matter of controversial discussions. Today, regarding these things retrospectively together with new results, important inconsistencies no longer exist.

Influence of Assay Conditions and Chemical Modification. It is the aim of all investigations on aminoacyl-tRNA synthetases to obtain information about the function and the mechanism with which these enzymes work under physiological conditions in the cell. Unfortunately, nobody can simulate the exact physiological conditions in his enzyme assay. Normally, after preparation of an enzyme, biochemists look for assay conditions under which they get the highest enzyme activity or which they just like most and carry out their experiments in this assay. In this way research groups apply to their experiments different temperatures, buffers, and additional compounds. Conclusions on enzyme functions in the cell are difficult, and even comparisons of results reported by different

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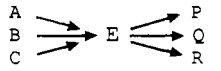
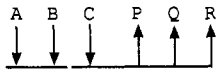
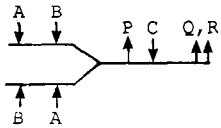
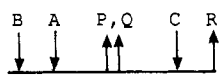
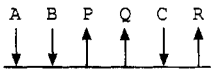
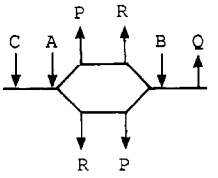
Table I: Different Orders of Substrate Additions and Product Releases and k_{cat} and K_m Values for Isoleucyl-tRNA Synthetase from Yeast[a]

order	nomenclature	conditions[b]	k_{cat} (s^{-1})	K_m (mM)
	sequential random Ter-Ter	pH 6.5	0.47	0.03
	sequential ordered Ter-Ter	pH 7.65	0.83	0.02
		pH 8.6	0.52	0.02
		pH 7.65 + EF-Tu·GTP	0.28	0.02
		pH 7.65 + EF-Tu·GTP + PPase	0.23	0.05
	random Bi-Uni Uni-Bi ping-pong	pH 7.65 + PPase	1.40	0.04
	Bi-Bi Uni-Uni ping-pong	pH 7.65 + EF-Tu·GTP + spermine	0.098	0.02
		pH 7.65 + EF-Tu·GTP + spermine (KCl lower)	0.135	0.008

[a] A = ATP, B = isoleucine, C = $tRNA^{Ile}$-C-C-A, P = pyrophosphate, Q = Ile-$tRNA^{Ile}$-C-C-A, and R = AMP; nomenclature according to Cleland (1963, 1970). [b] For complete conditions, see Freist and Sternbach (1984) and Freist et al. (1985).

Table II: Order of Substrate Addition and Product Release in Aminoacylation of Modified $tRNA^{Ile}$-C-C-N for Isoleucyl-tRNA Synthetase from Yeast[a]

$tRNA^{Ile}$-C-C-N	order	nomenclature	k_{cat} (s^{-1})	K_m (mM)
$tRNA^{Ile}$-C-C-3′-dA		Bi-Bi Uni-Uni ping-pong	0.2	0.002
$tRNA^{Ile}$-C-C-A(3′-NH_2)		Bi-random-Bi Uni-Uni ping-pong	0.1	0.006

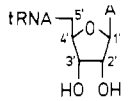
[a] A = ATP, B = isoleucine, C = $tRNA^{Ile}$-C-C-N, P = pyrophosphate, Q = Ile-$tRNA^{Ile}$-C-C-N, and R = AMP; nomenclature of orders according to Cleland (1963, 1970); k_{cat} values and K_m values of isoleucine obtained under standard conditions (Freist & Cramer, 1983).

research groups may be impossible.

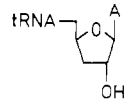
The influence of assay conditions on the aminoacylation pathway may be illustrated by results obtained with isoleucyl-tRNA synthetase from bakers' yeast, a single-chain enzyme with a molecular weight of 123 000 (Freist & Sternbach, 1984; Freist et al., 1985; Englisch et al., 1987). Four different orders of substrate additions and product releases were found under eight different reaction conditions by initial rate kinetic analyses (Table I). k_{cat} values changed in the range of 0.098–1.4 s^{-1} and K_m values of isoleucine in the range of 0.008–0.05 mM. Addition of inorganic pyrophosphatase, elongation factor EF-Tu·GTP, and spermine can be regarded as a stepwise, but still insufficient, approximation to physiological conditions. The results show that experiments carried out with unmodified natural substrates in the "standard reaction mixture" at pH 7.65 may differ considerably from results obtained under physiological conditions under which additional compounds of the cytosol are present.

It should be emphasized that changes in the order of substrate additions and product releases must not affect the chemical pathway of the reaction. In my opinion the adenylate mechanism must be used by the enzyme in all cases, as will be suggested below in two hypothetical schemes of the catalytic cycle. Even for the curious Bi-Bi Uni-Uni ping-pong order [for nomenclature, see Cleland (1963, 1970)] in which aminoacylated $tRNA^{Ile}$-C-C-A is released before addition of free $tRNA^{Ile}$-C-C-A, a catalytic cycle with adenylate formation can be formulated if one assumes that the enzyme has more than one binding site for the substrates and products.

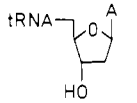
In many mechanistic studies on aminoacyl-tRNA synthetases, modified tRNAs were used as tools [compilations in Sprinzl and Cramer (1978), Chladek and Sprinzl (1985), and Freist (1988)]. As described above, orders of substrate additions and product releases were found to be very sensitive for changes of reaction conditions in the case of isoleucyl-tRNA synthetase from yeast. In Table II are shown the orders of substrate additions and product releases observed in aminoacylation of two modified $tRNA^{Ile}$s, namely, $tRNA^{Ile}$-C-C-3′-dA and $tRNA^{Ile}$-C-C-A(3′-NH_2).



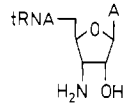
tRNA-C-C-A



tRNA-C-C-3′-dA



tRNA-C-C-2′-dA



tRNA-C-C-A(3′-NH_2)

These orders deviate from those obtained with natural $tRNA^{Ile}$-C-C-A under standard conditions. However, these deviations are within the scope of changes that are also observed by variation of buffer conditions or addition of natural compounds of the cytosol such as elongation factor EF-Tu·GTP. In both cases the same care has to be taken in conclusions on enzyme mechanisms occurring in the cytosol.

Enzyme–Substrate Interaction. Substrate analogues have often been tested for their properties as substrates or inhibitors in the aminoacylation reaction [e.g., von der Haar and Cramer (1978) and Freist et al. (1981)]. From inhibition patterns of dead-end inhibitors, hints on the number of substrate binding

sites were observed in several cases; further conclusions could be drawn concerning the essential atom groups of the substrates. The results agree in part with investigations obtained by physical methods.

For phenylalanyl-tRNA synthetase from yeast [subunit structure $\alpha_2\beta_2$, M_r = 276000; compilation of subunit structures and M_r values in Joachimiak and Barciszweski (1980)] it was found by ultracentrifugation analysis, fluorescence titrations, and fast kinetic techniques that the enzyme binds two molecules of $tRNA^{Phe}$-C-C-A (Krauss et al., 1976, 1975). When chemically modified $tRNA^{Phe}$s were tested in the aminoacylation reaction, actually some noncompetitive inhibitors of the mixed type were found, indicating a second tRNA binding site (von der Haar & Gaertner, 1975). In this case, results obtained with chemically modified compounds were consistent with results obtained by physical methods.

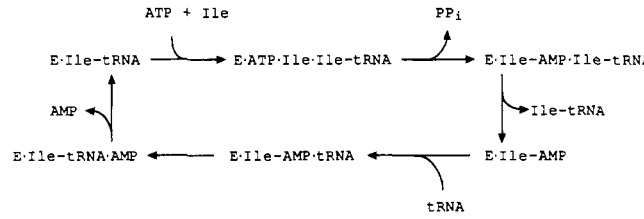
Differing results were obtained with isoleucyl-tRNA synthetases from yeast and *Escherichia coli* K12 (subunit structures α_1, M_r = 123000 and 110000). It was concluded from equilibrium partition or dialysis experiments that one enzyme molecule binds one molecule each of its three substrates (Fersht & Kaethner, 1976a; Berthelot & Yaniv, 1970; Yarus & Berg, 1969; Hustedt & Kula, 1977; Hustedt et al., 1977). Burst experiments with ^{32}P-labeled ATP and filtration of mixtures containing enzyme, labeled ATP, and isoleucine on nitrocellulose filters exhibited also a binding stoichiometry of one molecule per enzyme molecule for isoleucyladenylate, the intermediate after the first reaction step (Fersht & Kaethner, 1976a; Hustedt et al., 1977; Norris & Berg, 1964).

However, when modified tRNAs were tested as inhibitors of the aminoacylation reaction, classical noncompetitive inhibition was observed (von der Haar & Cramer, 1978), results that can only be explained either by the presence of a second binding site for tRNA on one enzyme molecule or by dimerization of the synthetase during catalytic action. In fact, polymerization phenomena could be observed with the enzyme from *E. coli* B (structure α_1, M_r = 112000) in equilibrium sedimentation experiments (Baldwin & Berg, 1966).

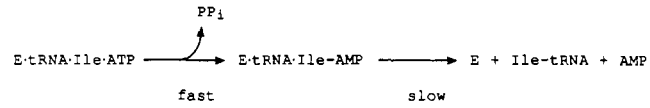
As will be mentioned below, the differing results may perhaps be due to different pathways of aminoacylation depending on assay conditions.

The first opportunity to compare conclusions on essential parts of the ATP molecule made from substrate specificity studies (Freist et al., 1976; Freist & Sternbach, 1988) with results obtained from X-ray analysis was when the structure of the tyrosyl-tRNA synthetase–tyrosyladenylate complex (subunit structure α_2, M_r = 88000) was solved and several hydrogen bonds between amino acids of the enzyme and tyrosyladenylate were postulated (Irwin et al., 1976; Rubin & Blow, 1981; Fersht et al., 1984; Fersht, 1987). As followed from substrate specificity with regard to ATP analogues, tyrosyl-tRNA synthetases check the hydroxyl groups of the ribose moiety, the conformation at the glycosyl bond, and especially the amino group in position 6 of the adenine part. The results of X-ray analysis agree with these findings except those concerning the adenine base. Whereas no specific interactions between the 6-amino group of the adenine base and the enzyme could be detected in the crystals, tyrosyl-tRNA synthetases do not accept an ATP analogue lacking this group as substrate in the aminoacylation reaction. The specificity for an intact, unmodified adenine moiety shows that during the catalytic cycle the enzyme must check the ATP molecule in a different conformation than found in the crystal, which makes it possible for the enzyme to discriminate ATP from, for example, GTP in the cytosol. Both methods give results that complement one another.

Postulation of Catalytic Cycles. On the basis of binding studies and comparison of the rate constants for the catalytic process, the first catalytic cycle was proposed in 1969 (Yarus & Berg) for isoleucyl-tRNA synthetase from *E. coli* B. In this reaction scheme release of the acylated tRNA is the rate-determining step; for the complete aminoacylation reaction a rate constant of 0.05 s^{-1} was found.



The enzyme from *E. coli* K12, as studied by pulsed quenched-flow techniques, has a different rate-determining step (Fersht & Kaethner, 1976; Fersht & Jakes, 1975): Transfer of the activated amino acid to the tRNA is crucial for the overall rate of the aminoacylation (k = 1.46 s^{-1}).



As shown in Table I for isoleucyl-tRNA synthetase from yeast, turnover numbers in the range of 0.1–1.4 s^{-1} were found depending on reaction conditions. To accommodate all the different results that emerged in studies using only physical methods, one can only speculate. For example, the enzyme may act in two types of different catalytic cycles, one rate determined by dissociation of the enzyme–aminoacyl-tRNA complex and the other rate determined by the transfer step (Freist & Sternbach, 1989). Two model cycles that are consistent with a Ter-Ter order of substrate additions and product releases are shown in Scheme I.

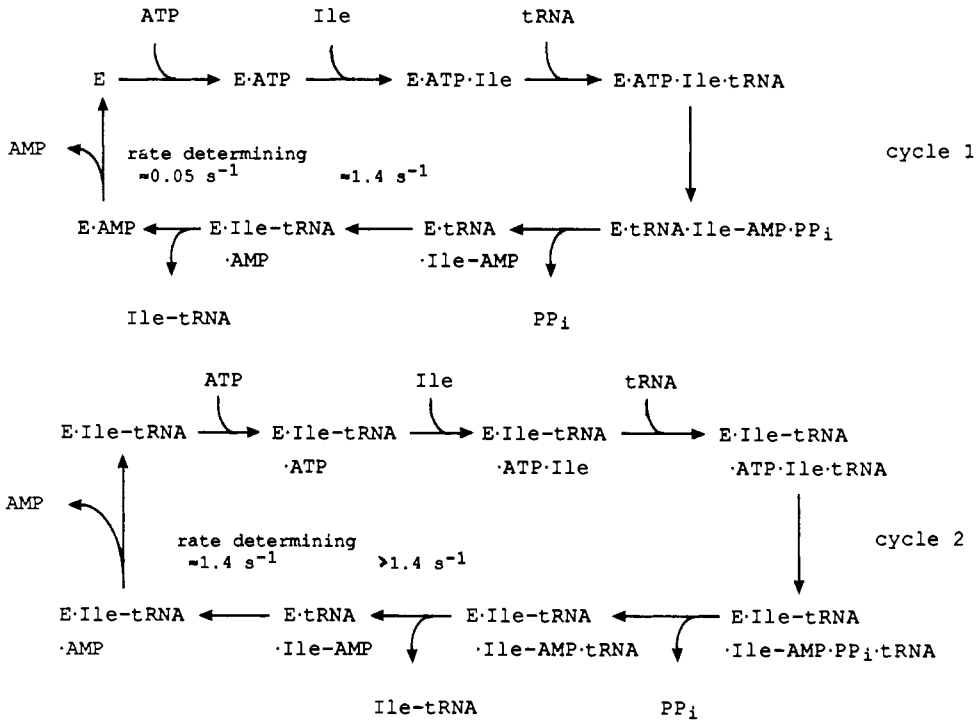
The two cycles differ in the number of substrate and product molecules attached to the enzyme. In cycle 1 only one tRNA molecule is complexed with the enzyme; cycle 2 involves complexes in which two tRNA molecules are bound to the enzyme. The protein "E" may be a monomer or a dimer. Similar cycles were also proposed for phenylalanyl- and valyl-tRNA synthetases (Thiebe, 1978; Kern & Gangloff, 1981).

Whereas in cycle 1 a low dissociation rate of about 0.05 s^{-1} may be valid for dissociation of a 1:1 complex of enzyme and tRNA, in cycle 2 from the complex of enzyme and two tRNA molecules tRNA may dissociate at higher rates and the transfer step may become rate determining.

In principle, these hypothetical cycles may be correct, but many details are only suspected because no experiments have been done to investigate the single steps of the cycles under appropriate assay conditions. Most probably, it is impossible to formulate a standard pathway, and the enzymes may function in different pathways according to the growth stages of the cell.

Site of Aminoacylation. An excellent example for elucidation of an important part of the aminoacylation mechanism using modified tRNAs was the determination of the aminoacylation site on the tRNA. It was found that aminoacyl-tRNA synthetases do not attach their cognate amino acids at the same position on tRNA molecules but show specificities for the 2′- or 3′-position (Sprinzl & Cramer, 1975; Fraser & Rich, 1975). Two different types of modified tRNAs were used: tRNA-C-C-2′-dA and tRNA-C-C-3′-dA, which lack one of the terminal hydroxyl groups, and tRNA-C-C-A(2′-

Scheme I



NH_2) and tRNA-C-C-A(3′-NH_2), in which one of the terminal hydroxyl groups is substituted by an amino group. The results obtained with both types of tRNAs were only slightly different and could be combined to a common and general picture (Sprinzl & Cramer, 1978). One could argue that specificity tests carried out with modified tRNAs may not be the same as acylation of natural tRNA under physiological conditions. This objection could be substantiated further because it was shown that orders of substrate additions and product releases are different in aminoacylation of $tRNA^{Ile}$-C-C-A, $tRNA^{Ile}$-C-C-3′-dA, and $tRNA^{Ile}$-C-C-A-(3′-NH_2) (Table II). However, the fact that very similar specificities for the 2′- or 3′-OH group were observed independent of the type of modification may indicate a general property of aminoacyl-tRNA synthetases that may also appear under conditions of protein biosynthesis. Furthermore, it was shown that the 2′,3′ specificity of the enzymes is only lost under extreme conditions at pH 9, at low salt concentrations, and after addition of organic solvents or inorganic pyrophosphatase (Igloi & Cramer, 1979; Freist & Cramer, 1983).

Early Hypotheses on Proofreading Mechanisms. When the amino acid specificities of aminoacyl-tRNA synthetases were determined under in vivo conditions, it was found that they were much higher than would be expected due to differences in binding energies between cognate and noncognate amino acids. For example, the error rate for incorporation of valine instead of isoleucine is 1:3000 in protein biosynthesis (Loftfield & Vanderjagt, 1972). Pauling had calculated a factor of only 4.3 by which incorporation of isoleucine may be favored rather than valine due to higher van der Waals forces (Pauling, 1958). Thus, special proofreading mechanisms were postulated by which the enzymes achieve their high discrimination rates.

The term "chemical proofreading" was created for a correction mechanism by which ester bonds between tRNAs and noncognate amino acids are hydrolyzed by the enzyme before release of the "wrong" product (von der Haar & Cramer, 1976). The hydrolytic capacity of some aminoacyl-tRNA synthetases was measured by incubation of aminoacyl-tRNAs with their cognate enzyme. Val-$tRNA^{Ile}$-C-C-A was hydrolyzed 3–5 times faster than Ile-$tRNA^{Ile}$-C-C-A (von der Haar & Cramer, 1976; Freist & Sternbach, 1989), proposing a "posttransfer proofreading factor" of about 3, a value that was also found later by other methods (Freist et al., 1988). Aminoacyl esters of tRNAs with a 2′- or 3′-deoxyadenosine instead of adenosine as terminal nucleoside were hydrolyzed 10–300 times slower than esters of the natural tRNA. As a rationale for the observation that two hydroxyl groups are essential for effective enzymic hydrolysis of aminoacyl-tRNA esters, it was assumed that hydrolysis takes place only when the aminoacyl moiety has migrated from the OH group to which it is initially attached to the nonaccepting OH. Thus, there would be one OH responsible for acylation and one for hydrolysis (von der Haar & Cramer, 1976; Fersht & Kaethner, 1976b).

According to new investigations (Freist & Sternbach, 1989), it is probable that higher stability of aminoacyldeoxy-tRNAs is due to a simple neighboring group effect of the OH groups as was found for cyclopentanediol esters in chemical hydrolysis (Bruice & Fife, 1962). The nonenzymic hydrolysis rate of cyclopentyl acetate is enhanced by a factor of about 30 by a hydroxyl group in a neighboring position, and this is also the order of magnitude by which aminoacyl-$tRNA^{Ile}$-C-C-A is hydrolyzed faster than aminoacyl-$tRNA^{Ile}$-C-C-dA.

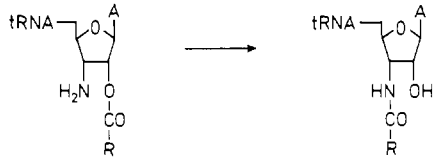
In our recent work (Freist & Sternbach, 1989) we have prepared isoleucyl and valyl esters of $tRNA^{Ile}$-C-C-3′-dA and $tRNA^{Ile}$-C-C-2′-dA. The esters of both tRNAs were stable against enzymic hydrolysis by isoleucyl-tRNA synthetase from bakers' yeast to the same extent, so that at least for this enzyme no preference for one of the two terminal hydroxyl groups in hydrolysis can be postulated.

From kinetic investigations of isoleucyl-tRNA synthetase from *E. coli* K12, it was concluded that rejection of valine in aminoacylation of $tRNA^{Ile}$-C-C-A is mainly achieved by an "editing mechanism" on the pretransfer level (Fersht, 1977a): The enzyme-bound adenylate of noncognate valine is hydrolyzed in a pretransfer proofreading step, and posttransfer hydrolysis of Val-$tRNA^{Ile}$-C-C-A only occurs to a minor extent and is considered to be a "mopping up" step.

At first glance chemical proofreading and the editing mechanism seemed in contrast. However, some years later it was shown more clearly that aminoacyl-tRNA synthetases use both types of error corrections but to different extents (Lin et al., 1984; Freist et al., 1985, 1988; Cramer & Freist, 1987; Freist & Sternbach, 1988).

Two-Step Proofreading. The contribution of proofreading processes to specificity of the aminoacyl-tRNA synthetases can be determined by measuring AMP formation stoichiometry of the aminoacylation reaction. For each misactivated, mistransferred, and rejected noncognate amino acid, one additional molecule of ATP must be hydrolyzed. For example, for mischarging of $tRNA^{Ile}$-C-C-A with valine by isoleucyl-tRNA synthetase from *E. coli* B, a total of 270 AMP molecules formed per molecule of Val-$tRNA^{Ile}$-C-C-A was found in the presence of elongation factor EF-Tu·GTP (Hopfield et al., 1976); a similar value of 192 was obtained for the yeast enzyme under the same conditions (Freist et al., 1985); under standard conditions the numbers were 1490 and 828 (Freist et al., 1988; Cramer et al., 1979). In the first stages of these investigations authors assigned the reaction steps responsible for the high AMP formation rates exclusively either to a pretransfer proofreading event (Hopfield et al., 1976) or to a posttransfer process (Cramer et al., 1979).

After several of those misleading assumptions, distribution of AMP formation to pre- and posttransfer proofreading steps could be established when AMP formation rates in aminoacylation of modified tRNAs, especially $tRNA^{Ile}$-C-C-A(3′-NH_2), were measured (Freist et al., 1987, 1988). When this tRNA species is aminoacylated, the final products of the reaction are not esters but amides because the aminoacyl esters that are initially generated rearrange by spontaneous intramolecular aminolysis to aminoacylamides [compare discussion in Chladek and Sprinzl (1985)].



These compounds are stable against enzymic hydrolysis (Fraser & Rich, 1975; Freist & Sternbach, 1988b), and thus proofreading can only be achieved on the pretransfer level when this type of modified tRNA is aminoacylated. A falsification of pretransfer AMP formation rates by partial enzymic hydrolysis before acyl migration can be ruled out by considering transacylation rates. For example, $tRNA^{Ile}$-C-C-A is aminoacylated at the 2′-OH group. For transacylation of 2′-*O*-aminoacyladenosines to the 3′-O derivative, rates of 3–11 s^{-1} have been measured, values considerably higher than those of enzymic hydrolysis found with the yeast enzyme (von der Haar & Cramer, 1976; Freist & Sternbach, 1989). Unfortunately, transacylation rates of 2′-*O*-aminoacyl-3′-deoxy-3′-aminoadenosines have not been measured, but according to chemical knowledge this reaction must run much faster, and lack of such measurements may be due to the difficulties in preparing such spontaneously isomerizing compounds. As an analogous reaction, formation of peptide bonds at the ribosome may be mentioned. It has been shown that, without enzymic transferase activity, when the ribosome functions only as template, aminolysis can take place at rates of at least 15–20 s^{-1} (Nierhaus et al., 1980).

As a control that the structure of the tRNA does not influence pretransfer proofreading, in some experiments with isoleucyl-tRNA synthetase from yeast $tRNA^{Ile}$-C-C-A(3′-NH_2) was substituted by $tRNA^{Ile}$-C-C-2′-dA. This latter species lacks the accepting OH group and cannot be aminoacylated; thus AMP formation by proofreading can also only be achieved with the aminoacyladenylate. It turned out that pretransfer proofreading is not dependent on the tRNA structure and that pretransfer proofreading factors determined for one tRNA species are also valid for experiments with other tRNAs (Freist & Sternbach, 1989).

When AMP formation rates were measured in aminoacylation of $tRNA^{Ile}$-C-C-A(3′-NH_2), considerably lower numbers of AMP generated per molecule of Val-$tRNA^{Ile}$-C-C-A(3′-NH_2) were found than in acylation of $tRNA^{Ile}$-C-C-A. These values were, under standard conditions, 111 for the yeast enzyme and 24 for the enzyme from *E. coli* MRE 600 (α_1, M_r = 102 000) (Freist et al., 1987). Divided by the number of AMP molecules generated during aminoacylation with the cognate substrate, pretransfer proofreading factors of Π_1 = 51 and Π_1 = 22 are obtained. These factors show how many times more the noncognate substrate valine is rejected by hydrolysis of the adenylate than the cognate one.

The pretransfer proofreading factor Π_1 is multiplied by the posttransfer factor Π_2 to give the overall proofreading factor Π' (compare Scheme II): $\Pi' = \Pi_1\Pi_2$. Because the overall factor can be determined in aminoacylation of $tRNA^{Ile}$-C-C-A, Π_2 can be calculated as $\Pi_2 = \Pi'/\Pi_1$. In the case of valine posttransfer proofreading factors of 3 and 62 are obtained. In case of the yeast enzyme (Π_1 = 51, Π_2 = 3) pretransfer proofreading is clearly the main correction step, whereas posttransfer proofreading plays a minor role. Π_2 values calculated for the other 18 noncognate amino acids are even lower than 3, confirming the assumption that posttransfer proofreading *can* be only a mopping up step or can even be completely neglected (Fersht, 1977a). However, for rejection of valine by isoleucyl-tRNA synthetase from *E. coli*, the misacylation reaction for which the term mopping up was created, we found more post- than pretransfer proofreading (Π_1 = 22, Π_2 = 62). This observation seems to be consistent with the higher rates of enzymic hydrolysis of Val-$tRNA^{Ile}$-C-C-A by this enzyme (Fersht, 1977a) than found for the yeast synthetase (von der Haar & Cramer, 1976; Freist & Sternbach, 1989). It should be mentioned that under standard assay conditions rejection of valine by isoleucyl-tRNA synthetase from *E. coli* is the only known case in which posttransfer hydrolysis rates are higher than pretransfer proofreading.

Summarizing the results on proofreading, it must be concluded that no general statement concerning distribution to pre- or posttransfer steps can be made. The structure of the misactivated amino acid seems to be decisive for this choice, and in many cases only pretransfer correction takes place. Furthermore, assay conditions also have an influence on the values of proofreading factors as will be discussed later.

Two-Step Initial Discrimination. When Pauling (1958) calculated the probabilities by which errors can occur in protein biosynthesis, he found a factor of 4.3 by which incorporation of alanine instead of glycine or isoleucine instead of valine is favored by higher van der Waals attraction energy. This factor is based on the difference of interaction of a methyl group and a hydrogen atom of 3.78 kJ/mol = 0.90 kcal/mol which causes a discrimination factor of exp ($900/RT$) = 4.3. Similar values were also obtained experimentally from investigation of antigen–antibody interactions (Pauling & Pressman, 1945).

A convenient method for measuring such differences in binding energies $\Delta\Delta G_b$ is given by the equation $(k_{cat}/K_m)_A/(k_{cat}/K_m)_B = \exp(-\Delta\Delta G_b/RT)$, which allows calculation of the difference in Gibbs free energies of binding from the kinetic

constants k_{cat} and K_m (Fersht, 1977b).

Because valine is misactivated by isoleucyl-tRNA synthetase, a $\Delta\Delta G$ value of about 13 kJ $\approx$ 3 kcal/mol could be calculated from k_{cat} and K_m values obtained with enzymes from *E. coli* in the pyrophosphate exchange reaction (Loftfield & Eigner, 1966; Fersht, 1977b). Very similar values were also measured later by other authors, and thus an incremental group binding energy of 3.4 kcal/mol was given for a methyl group (Fersht, 1977a; Fersht & Dingwall, 1979a,b; Fersht et al., 1980). As a reason for these higher values, Fersht (1981) discusses higher van der Waals forces due to preformed binding pockets and a dense package in the binding sites of aminoacyl-tRNA synthetases.

Supporting this idea, Hopfield and Yamane (1979) calculated that in an "ideally" arranged binding pocket 6–9 methyl equivalents could be tightly packed around the methyl group by which the isoleucine side chain is longer than the valine side chain and result in a total difference of Gibbs free energy of about 10.5 kJ/mol = 2.5 kcal/mol.

Surprisingly, when $tRNA^{Ile}$-C-C-A(3′-NH_2) was aminoacylated with isoleucine and valine, a difference of $\Delta\Delta G_{I_1}$ = 3.08 kJ/mol = 0.74 kcal/mol in Gibbs free energy was obtained (Freist et al., 1987, 1985), the same value that was calculated and measured by Pauling 30 years ago. For determination of this value it was taken into account that the discrimination factor $D_1 = (k_{cat}/K_m)_{Ile}/(k_{cat}/K_m)_{Val}$ [discrimination factors are called D_1 in aminoacylation of $tRNA^{Ile}$-C-C-A(3′-NH_2); they indicate how many times more the cognate substrate is converted than the noncognate one at the same concentration] must be the product of an initial discrimination factor I_1 and the pretransfer proofreading factor Π_1: $D_1 = I_1\Pi_1$. Thus, I_1 can be calculated as $I_1 = D_1/\Pi_1$ and the difference in Gibbs free energy of binding as $\Delta\Delta G_{I_1} = RT \ln I_1$.

However, in aminoacylation of $tRNA^{Ile}$-C-C-A higher differences in Gibbs free energy of 14.25 kJ/mol = 3.40 kcal/mol were observed (Freist et al., 1987, 1985). Presently, an exact explanation cannot be given for this phenomenon; plausible rationales exist but remain hypothetical. The higher difference in Gibbs free energy obtained in acylation of $tRNA^{Ile}$-C-C-A may indeed by due to a "more dense" package in the amino acid binding pocket. In acylation of $tRNA^{Ile}$-C-C-A(3′-NH_2) this "dense" package may not be achieved.

By rapid kinetic investigations complex formation of aminoacyl-tRNA synthetase and amino acid as well as of enzyme and tRNA has been found to be a two-step process (Holler & Calvin, 1972; Krauss et al., 1979, 1977). With modified tRNAs complex formation is reduced to a one-step process (Krauss et al., 1979, 1977). It seems reasonable to assume that a dense package in the amino acid binding pocket is achieved in a two-step process and that with modified tRNAs the binding site remains uncompleted.

As a working hypothesis one can assume that in the first step of amino acid binding different acids are discriminated by a factor I_1 and after a conformational change of the enzyme in the second step by a factor I_2 (compare Scheme II). Both factors are multiplied to an overall initial discrimination factor I'. The overall difference in Gibbs free energy of binding $\Delta\Delta G_{I'}$ is thus distributed to the two steps of initial discrimination: $\Delta\Delta G_{I'} = \Delta\Delta G_{I_1} + \Delta\Delta G_{I_2}$ = 3.08 + 11.17 = 14.25 kJ/mol, or 0.74 + 2.67 = 3.41 kcal/mol (Freist et al., 1987, 1988).

Similarly, as proofreading processes could be assigned to pre- and posttransfer steps by experiments with chemically modified tRNAs, these compounds showed that the initial discrimination of amino acids in their special binding pocket must also be a process that is more complex than thought before. Although no final theory could be postulated, a plausible hypothesis was obtained which is in accordance with physical measurements.

Overall Discrimination of Amino Acids. The factor of physiological relevance is the overall discrimination factor D, valid in aminoacylation of tRNA-C-C-A. As described above, it is determined by four subfactors, two initial discrimination factors and one pre- and one posttransfer proofreading factor:

$$D = I_1 I_2 \Pi_1 \Pi_2$$

Factor D is decisive for the quotient of velocities by which two amino acids are attached to the tRNA by the synthetase; e.g., for discrimination of isoleucine and valine the following equation is valid [see also Fersht (1977b)]:

$$v_{Ile}/v_{Val} = D[Ile]/[Val]$$

In detailed studies factors D of 19 noncognate amino acids have been calculated from k_{cat} and K_m values as $D = (k_{cat}/K_m)_{cognate}/(k_{cat}/K_m)_{noncognate}$ for isoleucyl-tRNA synthetases from yeast and *E. coli* MRE 600 as well as for tyrosyl-tRNA synthetase from yeast (Freist et al., 1988; Freist & Sternbach, 1988). Isoleucyl-tRNA synthetases show the highest D values for discrimination of valine (38 000 and 72 000); for most noncognate acids factors are in the range of 10 000–50 000, except for four amino acids which are only rejected with D values between 300 and 3000. D values determined for tyrosyl-tRNA synthetase are considerably higher, in a range from 30 000 to more than 500 000. Whereas isoleucyl-tRNA synthetases show the highest accuracy in rejection of valine, the amino acid most similar to the cognate substrate, tyrosyl-tRNA synthetase shows the lowest specificity in discrimination of the most similar substrate phenylalanine and the highest D values for the acids that deviate most from the tyrosine structure.

It should be mentioned that these D values are in the same order of magnitude as some values given earlier by other authors. For example, for discrimination between isoleucine and valine by isoleucyl-tRNA synthetase a factor of about 18 000 had been estimated (Hopfield et al., 1976); for rejection of tyrosine by phenylalanyl-tRNA synthetase a factor of 20 000 had been found by applying fast kinetic techniques (Lin et al., 1984). As also observed with the isoleucyl-tRNA synthetases from yeast and *E. coli*, tyrosyl-tRNA synthetase from *E. coli* seems to be more specific than the yeast enzyme. For discrimination between phenylalanine and tyrosine a 7-fold higher D value was calculated for the *E. coli* enzyme (Fersht et al., 1980).

Influence of Assay Conditions on Specificity and Energy Consumption. As shown in Table I, k_{cat} and K_m values observed for isoleucine as the cognate substrate are considerably changed by assay conditions. Because these values are decisive for discrimination between cognate and noncognate substrates, factors D should also be changed. This was indeed found when factors D were determined under different assay conditions for discrimination between isoleucine and valine in acylation of $tRNA^{Ile}$-C-C-A by the yeast enzyme (Freist et al., 1985). Applying the same conditions as given in Table I, factors D changed in the range from 2000 to 38 000. Remarkably, these variations were nearly exclusively caused by different k_{cat} and K_m values of the cognate substrate, whereas for unknown reasons kinetic constants of the noncognate substrate valine were nearly constant.

The question concerning which recognition steps are mainly influenced by assay conditions may be answered by comparison

Scheme II: Four-Step Recognition of Amino Acids

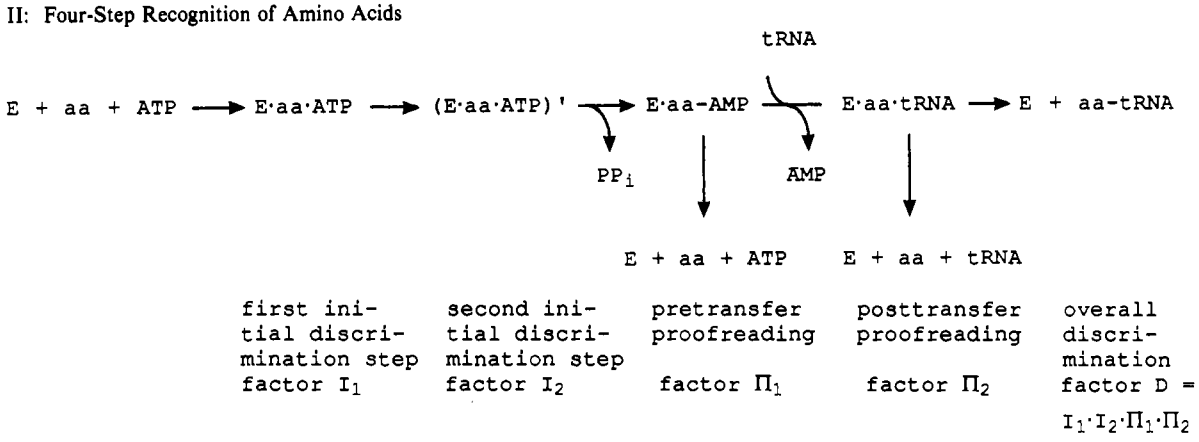


Table III: Discrimination Factors D and Subfactors I_1, I_2, Π_1, and Π_2 at Different pH Values Obtained for Discrimination of Valine in Aminoacylation of $tRNA^{Ile}$-C-C-A by Isoleucyl-tRNA Synthetase from Yeast (Freist et al., 1985)

pH	D	I_1	I_2	Π_1	Π_2
6.50	8000	3.1	31.4	27.7	3.0
7.65	38000	3.3	76.5	50.5	3.0
8.60	14000	3.1	53.0	35.3	2.4

of discrimination subfactors calculated from experiments with $tRNA^{Ile}$-C-C-A and $tRNA^{Ile}$-C-C-A(3'-NH_2) at different pH values (Freist et al., 1985). In Table III it is shown that for discrimination of valine by isoleucyl-tRNA synthetase initial discrimination factors I_1 and posttransfer proofreading factors Π_2 are approximately constant whereas initial discrimination factors I_2 and pretransfer proofreading factors Π_1 vary up to a factor of about 2. This means that the assumed conformational change of the enzyme which completes the amino acid binding site is pH dependent, as is the following hydrolytic pretransfer correction step. It must be further assumed that these different conformational states of the enzyme are somehow related to the different orders of substrate additions and product releases (Table I).

At present it is not clear whether other aminoacyl-tRNA synthetases exhibit variation of specificity to the same extent depending on assay conditions, but it may be already concluded that in yeast cells isoleucine may be incorporated into proteins with changing accuracy.

Another striking property that has until now only been observed with isoleucyl-tRNA synthetase prepared from commercial bakers' yeast was a very high ATP consumption. According to the overall reaction equation for esterification of the tRNA with the cognate substrate, one molecule of AMP should be generated. For isoleucyl-tRNA synthetase from *E. coli* B an AMP formation stoichiometry of 1.5 (Hopfield et al., 1976) and for that enzyme from *E. coli* MRE 600 an AMP formation stoichiometry of 1.1 (Freist et al., 1988) were found; for tyrosyl- and arginyl-tRNA synthetases from yeast (α_2, M_r = 80000; α_1, M_r = 73000) formation stoichiometries of 1.1 and 1.7 AMP/aminoacyl-tRNA have been found (Freist & Sternbach, 1988; Freist et al., in preparation). However, isoleucyl-tRNA synthetase from bakers' yeast needs 5.5 molecules of AMP per molecule of Ile-$tRNA^{Ile}$-C-C-A under standard conditions (Freist et al., 1985). Obviously, the enzyme prepared from the commercial yeast has lost its ability to recognize exactly its cognate substrate in the hydrolytic correction steps and thus wastes energy by hydrolyzing ATP. However, this high ATP consumption made it possible to observe more clearly ATP consumption dependent on assay conditions in acylation with the cognate substrate. Generally it turned out that high accuracy is connected with high energy consumption of the cognate as well as the noncognate reactions.

Structures of Substrate Binding Pockets. Important progress in understanding the function of single amino acid side chains of the aminoacyladenylate binding site was achieved when the crystal structure of the tyrosyladenylate–tyrosyl-tRNA synthetase complex was solved (Monteilhet & Blow, 1978; Blow & Brick, 1985) and site-directed mutagenesis work was carried out systematically to analyze the function of the substrate binding residues of the enzyme [compilation in Fersht (1987)]. Single amino acids involved in hydrogen bonding could be exchanged and thus Gibbs free energy values for single hydrogen bonds could be determined (Fersht, 1988). The side chain of the substrate tyrosine forms two hydrogen bonds to the enzyme by its hydroxyl group in the para position of the phenyl residue: one to an aspartate side chain and one to a tyrosyl moiety of the enzyme. Changing the tyrosyl residue of the enzyme into a phenylalanyl residue allowed a determination of a contribution of 2.1 kJ = 0.5 kcal/mol of Gibbs free energy to substrate binding due to the tyrosine–tyrosine hydrogen bond. Mutation of the aspartate moiety of the enzyme did result in an inactive enzyme (Fersht, 1987), and Gibbs free energy values of the second hydrogen bond could not be determined directly, but from other experiments a value of 14–18 kJ = 3.5–4.5 kcal/mol could be given for such a hydrogen bond between a charged and an uncharged group (Fersht et al., 1985).

These results could be partly confirmed by changing the substrate instead of single amino acids of the active site of the synthetase. Binding of amino acid side chains is mainly caused by hydrophobic interaction and additionally, as in the case of the cognate substrate tyrosine, by hydrogen bonds. Decisive for hydrophobic interactions are the free accessible surface areas of the amino acids; in the literature a mean energy value of 105 J/mol = 25 cal/mol is given for removal of an accessible surface of 0.01 nm^2 = 1 $Å^2$ from contact with water (Chothia, 1976, 1975, 1974).

When $\Delta\Delta G_{I_1}$ and $\Delta\Delta G_{I_2}$ values (calculated as $\Delta\Delta G_I = RT \ln I$) were plotted against accessible surface areas, linear relationships were observed in which 0.01 nm^2 = 1 $Å^2$ of accessible surface corresponds to 42–150 J/nm^2 or 10–30 cal/$Å^2$, values which are indeed in a range that must be expected for hydrophobic interaction (Freist et al., 1987, 1988).

From X-ray analysis of tyrosyl-tRNA synthetase it was also concluded that, by tyrosyladenylate formation and binding, some amino acid side chains in the vicinity of the binding site are shifted to other positions (Rubin & Blow, 1981; Monteilhet & Blow, 1978). This observation may correspond to some irregularities found in the linear relationships of $\Delta\Delta G$ values to accessible surface areas. If amino acid side chains of the

enzyme must be turned aside during substrate binding, parts of the hydrophobic interaction energies may be lost in those processes. A special "stopper model" was created for isoleucyl- and tyrosyl-tRNA synthetases which explains initial recognition of amino acids by hydrophobic interaction and removal of enzyme side chains called "stoppers" (Freist et al., 1988; Freist & Sternbach, 1988). In this stopper model the binding cavity of, e.g., isoleucine is restricted to the length of the isoleucine side chain by a bulky "stopper group". If an amino acid with a longer side chain, such as tryptophan, is bound to this site, the bulky stopper group is shifted to another position. The higher hydrophobic interaction energy gained by the longer side chain of tryptophan is thus compensated by the energy that is necessary for pushing aside the stopper group preventing a favored binding of tryptophan.

From $\Delta\Delta G_I$ plots the Gibbs free energy contributions of the two hydrogen bonds could also be estimated by which the tyrosine side chain is bound to the enzyme. Tyrosine deviates from linear relation of binding energy to its accessible surface area by 12.2 kJ = 2.91 kcal/mol and 6.3 kJ = 1.51 kcal/mol. These two values are in the same range as determined by site-directed mutagenesis methods for hydrogen bonds between an uncharged and a charged group and between two uncharged groups.

Conclusions. Although much progress has been made in the investigation of aminoacyl-tRNA synthetases, we are still far away from a complete understanding of the function and mechanism of these enzymes. As described above for recognition of amino acids, at least a four-step process must be assumed as shown in Scheme II. However, for the complete picture of catalysis this scheme must be combined to a multistep process with the catalytic cycles as shown above for the Ter-Ter substrate additions and product releases. At present this would be far too speculative. Today we have a general and rough knowledge on aminoacyl-tRNA synthetases, but many details are still missing.

Kinetic investigations have shown that the enzymes have many different active conformations and can act in different catalytic cycles. There may be a large population of active enzyme conformations. No conclusions can be made as to the nature of conformational differences at this time. Even if more X-ray structure analysis could be done, it would be difficult to elucidate these processes because in the crystals the molecules are in a "frozen" state favorable for crystallization. Probable action of enzymes like the aminoacyl-tRNA synthetases may reach the borders of "fundamental complex" processes (Cramer, 1979), which are very difficult to understand in all their details.

Registry No. Aminoacyl-tRNA synthetase, 9028-02-8.

References

Baldwin, A. N., & Berg, P. (1966) *J. Biol. Chem. 241*, 831–838, 839–845.

Berthelot, F., & Yaniv, M. (1970) *Eur. J. Biochem. 16*, 123–125.

Blow, D. M., & Brick, P. (1985) in *Biological macromolecules and assemblies. Vol. 2: Nucleic acids and interactive proteins* (Jurnak, F., & McPherson, A., Eds.) pp 443–469, Wiley, New York.

Bruice, T. C., & Fife, T. H. (1962) *J. Am. Chem. Soc. 84*, 1973–1979.

Chladek, S., & Sprinzl, M. (1985) *Angew. Chem. 97*, 377–398; *Angew. Chem., Int. Ed. Engl. 24*, 371–391.

Chothia, C. (1974) *Nature (London) 248*, 338–339.

Chothia, C. (1975) *Nature (London) 254*, 304–308.

Chothia, C. (1976) *J. Mol. Biol. 105*, 1–14.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137, 173–187, 188–196.

Cleland, W. W. (1970) *Enzymes (3rd Ed.) 2*, 1–65.

Cramer, F. (1979) *Interdiscip. Sci. Rev. 4*, 132–139.

Cramer, F., & Freist, W. (1987) *Acc. Chem. Res. 20*, 79–84.

Cramer, F., von der Haar, F., & Igloi, G. (1979) in *Transfer RNA: Structure, Properties, and Recognition*, pp 267–279, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Deutscher, M. P. (1967) *J. Biol. Chem. 242*, 1132–1139.

Englisch, U., Englisch, S., Markmeyer, P., Schischkoff, J., Sternbach, H., Kratzin, H., & Cramer, F. (1987) *Biol. Chem. Hoppe-Seyler 368*, 971–979.

Fersht, A. R. (1977a) *Biochemistry 16*, 1025–1030.

Fersht, A. R. (1977b) *Enzyme Structure and Mechanism*, Freeman, Reading, MA, and San Francisco, CA.

Fersht, A. R. (1981) *Proc. R. Soc. London, B 212*, 351–379.

Fersht, A. R. (1987) *Biochemistry 26*, 8031–8037.

Fersht, A. R. (1988) *Biochemistry 27*, 1577–1580.

Fersht, A. R., & Jakes, R. (1975) *Biochemistry 14*, 3350–3356.

Fersht, A. R., & Kaerthner, M. M. (1976a) *Biochemistry 15*, 818–823.

Fersht, A. R., & Kaethner, M. M. (1976b) *Biochemistry 15*, 3342–3346.

Fersht, A. R., & Dingwall, C. (1979a) *Biochemistry 18*, 2627–2631.

Fersht, A. R., & Dingwall, C. (1979b) *Biochemistry 18*, 1238–1245.

Fersht, A. R., Shindler, J. S., & Tsui, W.-C. (1980) *Biochemistry 19*, 5520–5524.

Fersht, A. R., Shi, I.-P., Wilkinson, A. I., Blow, D. M., Carter, P., Waye, M. M. Y., & Witner, G. P. (1984) *Angew. Chem. 96*, 455–462; *Angew. Chem., Int. Ed. Engl. 23*, 467–474.

Fersht, A. R., Shi, I.-P., Knill-Jones, I., Lowe, D. M., Wilkinson, A. I., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London) 314*, 235–238.

Fraser, T. H., & Rich, A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3044–3048.

Freist, W. (1988) *Angew. Chem. 100*, 795–811; *Angew. Chem., Int. Ed. Engl. 27*, 773–788.

Freist, W., & Cramer, F. (1983) *Eur. J. Biochem. 131*, 65–80.

Freist, W., & Sternbach, H. (1984) *Biochemistry 23*, 5742–5752.

Freist, W., & Sternbach, H. (1988) *Eur. J. Biochem. 177*, 425–433.

Freist, W., & Sternbach, H. (1989) *Eur. J. Biochem. 178*, 595–602.

Freist, W., von der Haar, F., Faulhammer, F., & Cramer, F. (1976) *Eur. J. Biochem. 66*, 493–497.

Freist, W., Sternbach, H., & Cramer, F. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1247–1254.

Freist, W., Sternbach, H., & Cramer, F. (1982) *Eur. J. Biochem. 128*, 315–329.

Freist, W., Pardowitz, I., & Cramer, F. (1985) *Biochemistry 24*, 7014–7023.

Freist, W., Sternbach, H., & Cramer, F. (1987) *Eur. J. Biochem. 169*, 33–39.

Freist, W., Sternbach, H., & Cramer, F. (1988) *Eur. J. Biochem. 173*, 27–34.

Holler, E., & Calvin, M. (1972) *Biochemistry 11*, 3741–3752.

Hopfield, J. J., & Yamane, T. (1979) in *Ribosomes: Structure, function and genetics* (Chambliss, G., Craven, G. R., Davies, I., Davis, K., Kahna, L., & Nomura, M., Eds.) pp

585–591, University Park Press, Baltimore, MD.
Hopfield, J. J., Yamane, T., Yue, V., & Coutts, S. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1164–1168.
Hustedt, H., Flossdorf, J., & Kula, M.-R. (1977) *Eur. J. Biochem. 74*, 199–202.
Igloi, G. L., & Cramer, F. (1978) *FEBS Lett. 90*, 97–102.
Irwin, M. I., Nyborg, I., Reid, B. R., & Blow, D. M. (1976) *J. Mol. Biol. 105*, 577–586.
Joachimiak, A., & Barciszewski, J. (1980) *FEBS Lett. 119*, 201–211.
Kern, D., & Gangloff, I. (1981) *Biochemistry 20*, 2065–2075.
Kisselev, L. L., & Favorova, O. O. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 40*, 141–238.
Kovaleva, G. K., Holmuratov, E. G., & Kisselev, L. L. (1983) *FEBS Lett. 151*, 79–82.
Krauss, G., Pingoud, A., Boehme, D., Riesner, D., Peters, F., & Maass, G. (1975) *Eur. J. Biochem. 55*, 517–529.
Krauss, G., Riesner, D., & Maass, G. (1976) *Eur. J. Biochem. 68*, 81–93.
Krauss, G., Riesner, D., & Maass, G. (1977) *Nucleic Acids Res. 4*, 2253–2262.
Krauss, G., von der Haar, F., & Maass, G. (1979) *Biochemistry 18*, 2253–2262.
Lin, S. X., Baltzinger, M., & Remy, P. (1984) *Biochemistry 23*, 4109–4116.
Loftfield, R. B. (1972) *Prog. Nucleic Acid. Res. Mol. Biol. 12*, 87–128.
Loftfield, R. B., & Eigner, E. A. (1966) *Biochim. Biophys. Acta 130*, 426–448.
Loftfield, R. B., & Eigner, E. A. (1969) *J. Biol. Chem. 244*, 1746–1754.
Loftfield, R. B., & Vanderjagt, M. A. (1972) *Biochem. J. 128*, 1353–1356.
Lövgren, T. N. E., Heinonen, J., & Loftfield, R. B. (1975) *J. Biol. Chem. 250*, 3854–3860.
Monteilhet, C., & Blow, D. M. (1978) *J. Mol. Biol. 122*, 407–417.
Nierhaus, K. H., Schulze, H., & Cooperman, B. S. (1980) *Biochem. Int. 1*, 185–192.
Norris, A. T., & Berg, P. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 330–337.
Parfait, R., & Grosjean, H. (1972) *Eur. J. Biochem. 30*, 242–249.
Pauling, L. (1958) *Festschr. Prof. Dr. Arthur Stoll Siebzigsten Geburtstag, 1957*, 597–602.
Pauling, L., & Pressman, D. (1945) *J. Am. Chem. Soc. 67*, 1003–1012.
Rubin, J., & Blow, D. M. (1981) *J. Mol. Biol. 145*, 489–500.
Söll, D., & Schimmel, P. R. (1974) *Enzymes (3rd Ed.) 10*, 489–538.
Sprinzl, M., & Cramer, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3049–3053.
Sprinzl, M., & Cramer, F. (1978) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 1–69.
Thiebe, R. E. (1975) *FEBS Lett. 60*, 342–345.
Thiebe, R. E. (1978) *Nucleic Acids Res. 5*, 2055–2071.
Thiebe, R. E. (1983) *Eur. J. Biochem. 130*, 525–528.
von der Haar, F., & Gaertner, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1378–1382.
von der Haar, F., & Cramer, F. (1976) *Biochemistry 15*, 4131–4138.
von der Haar, F., & Cramer, F. (1978) *Biochemistry 17*, 3139–3145.
Yarus, M., & Berg, P. (1969) *J. Mol. Biol. 42*, 171–189.