# A Single Base Pair Affects Binding and Catalytic Parameters in the Molecular Recognition of a Transfer RNA†

Soon Jae Park, Ya-Ming Hou, and Paul Schimmel*

*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

*Received October 25, 1988; Revised Manuscript Received January 18, 1989*

ABSTRACT: A single $G^3 \cdot U^{70}$ base pair in the acceptor helix is a major determinant of the identity of an alanine transfer RNA. Alteration of this base pair to A·U or G·C prevents aminoacylation with alanine. We show here that, at approximate physiological conditions (pH 7.5, 37 °C), high concentrations of the mutant $A^3 \cdot U^{70}$ species do not inhibit aminoacylation of a wild-type alanine tRNA. The observation suggests that, under these conditions, the $G^3$ to $A^3$ substitution increases $K_m$ for tRNA by more than 30-fold. Other experiments at pH 7.5 show that no aminoacylation of $A^3 \cdot U^{70}$, $G^3 \cdot C^{70}$, or $U^3 \cdot G^{70}$ mutant tRNAs occurs with substrate levels of enzyme. This suggests that $k_{cat}$ for these mutant tRNAs is sharply reduced as well and that the catalytic defect is not due to slow release of charged mutant tRNAs from the enzyme. Investigations were also done at pH 5.5, where association of tRNAs with synthetases is generally stronger and where binding can be conveniently measured apart from aminoacylation. Under these conditions, the binding of the $A^3 \cdot U^{70}$ and $G^3 \cdot C^{70}$ species is readily detected and is only 3–5-fold weaker than the binding of the wild-type tRNA. Although the $A^3 \cdot U^{70}$ species was demonstrated to compete with the wild-type tRNA for the same site on the enzyme, no aminoacylation could be detected. Thus, even when conditions are adjusted to obtain strong competitive binding, a sharp reduction in $k_{cat}$ prevents aminoacylation of a $tRNA^{Ala}$ species with a substitution at position 3·70.

The recognition of transfer RNAs by aminoacyl-tRNA synthetases determines the rules of the genetic code whereby amino acids are assigned to specific nucleotide triplets. These triplets are the anticodons within tRNAs. The sites recognized by synthetases determine the identities of tRNAs, and recent research suggests that the locations of these sites vary from one tRNA species to another (Normanly et al., 1986; Hou & Schimmel, 1988; McClain & Foss, 1988; Muramatsu et al., 1988; Schulman & Abelson, 1988; Rogers, & Soll, 1988; Schulman & Pelka, 1988; Sampson et al., 1989; Francklyn & Schimmel, 1989). In some cases the anticodon itself may be a major determinant for identity while in other tRNAs it is of little or no significance. As an example of the latter, a complete substitution of the anticodon of an alanine tRNA yields a species that efficiently aminoacylates with alanine (Hou & Schimmel, 1988). For this tRNA, a single G·U base pair in the amino acid acceptor helix is a major determinant of the identity. Alteration of this $G^3 \cdot U^{70}$ base pair to $G^3 \cdot C^{70}$ or to $A^3 \cdot U^{70}$ prevents aminoacylation with alanine (Hou & Schimmel, 1988). Transfer of this base pair into a $tRNA^{Cys}$ (Hou & Schimmel, 1988) or a $tRNA^{Phe}$ (Hou & Schimmel, 1988; McClain & Foss, 1988) species enables each of these tRNAs to be aminoacylated with alanine. Moreover, a microhelix comprised of just seven base pairs can be aminoacylated with alanine, when the base pair that is analogous to G3·U70 is present (Francklyn & Schimmel, 1989).

The recognition of tRNAs is distinguished from that of other systems of protein–nucleic acid recognition, such as the protein–DNA complexes associated with gene regulatory proteins. In the latter cases, the small dissociation constants (which can be on the order of 1 pM) for the specific complexes can be sufficient to provide a high degree of discrimination between different nucleotide sequence elements (Pabo & Sauer, 1984). The long lifetimes of these complexes contrast with the much higher $K_m$'s for tRNAs in the aminoacylation reaction (typically about 1 $\mu$M or less at pH 7.5). These relatively high $K_m$ values facilitate the rapid product release and turnover of the enzymes during the aminoacylation reaction, but they also limit the degree to which specificity can be achieved through binding alone.

We report here an investigation of the interaction of alanyl-tRNA synthetase with wild-type and mutant $tRNA^{Ala}$ species that are altered at the critical 3 and 70 positions. In a previous report, we demonstrated that the $G^3 \cdot U^{70}$ and $A^3 \cdot U^{70}$ $tRNA^{Ala}$ species were not aminoacylated by catalytic amounts of alanyl-tRNA synthetase under conditions where aminoacylation of the wild-type tRNA species is readily observed. The question we sought to address was whether this defect in aminoacylation, which is caused by substitution of a single nucleotide, is due to an effect on binding or catalytic rate parameters, or both. To investigate this question, experiments were conducted at pH 7.5, 37 °C, which provides an approximation of the physiological environment, and also at pH 5.5, which provides an environment where binding of tRNA to enzyme can be conveniently measured independently of aminoacylation. The results obtained demonstate that both $K_m$ and $k_{cat}$ parameters play a role in enabling alanyl-tRNA synthetase to discriminate against the single-base mutant tRNA species and that, even when bound to the catalytic site, the sharply lowered $k_{cat}$ for the mutant species is sufficient to prevent detectable aminoacylation with alanine.

## Materials and Methods

*Materials. Escherichia coli* $tRNA^{Ala/UGC}$ was purchased from Subriden RNA (Rolling Bay, WA) and further purified by electrophoresis on a 12% polyacrylamide–7 M urea gel. Alanyl-tRNA synthetase was purified by Dr. Kelvin Hill in this laboratory according to a method described elsewhere

† Supported by National Institutes of Health Grants GM 15539 and GM 37641.

* To whom correspondence should be addressed.

0006-2960/89/0428-2740$01.50/0 

(Regan, 1986), and the concentration of the enzyme was determined by active-site titration (Fersht et al., 1975). Thin-layer chromatography plates (coated with the fluorescent indicator $60F_{254}$) and a $C_4$ reverse-phase column were purchased from Merck and Vydac, respectively. Calf intestine alkaline phosphatase and $tRNA^{fMet}$ were obtained from Boehringer Mannheim, polynucleotide kinase was from New England Biolabs, and RNase- and DNase-free bovine serum albumin was from Pharmacia LKB Biotechnology Inc. Du Pont–New England Nuclear provided [$\gamma$-$^{32}$P]ATP.

*Transfer RNA Preparation.* The wild-type suppressor $tRNA^{Ala/CUA}$ and the mutant suppressor tRNAs were purified from cultures of a *recA* version of strain FTP3689 carrying constructs of plasmid pGFIB:Ala (Hou & Schimmel, 1988). Crude tRNAs were prepared according to the method described in Hou and Schimmel (1988). The mixture of tRNAs was separated from ribosomal RNA and cellular DNA by Sephadex G-100 gel filtration with a buffer of 10 mM sodium acetate (pH 5.5), 0.15 M NaCl, 10 mM $MgCl_2$, and 5 mM EDTA. The tRNA fraction was collected and precipitated by ethanol. The wild-type $tRNA^{Ala/CUA}$ and the mutant suppressor $tRNA^{Ala}$ species were separated from others by nondenaturing 12% polyacrylamide gel electrophoresis in TBE buffer.[1] The bands containing the desired tRNAs were excised after ethidium bromide staining and eluted in the gel elution buffer (Wurst et al., 1978). For kinetic analysis, tRNAs were further purified by electrophoresis on a 12% polyacrylamide–7 M urea gel. The tRNAs were visualized by first placing the gel on a TLC plate that was coated with a fluorescent indicator and then exposing the gel to UV light. The main band that corresponds to the overproduced $tRNA^{Ala}$ species was cut and eluted from the gel. The aminoacylation assay was used to test the activity of the wild-type $tRNA^{Ala/CUA}$ and $tRNA^{Ala/UGC}$.

In the case of the suppressor$^-$ mutant tRNAs, the purity was tested by $C_4$ reverse-phase (0.45 × 25 cm) HPLC (Zhang et al., 1986). The tRNA was dissolved in 1 M sodium formate (or 1 M sodium acetate), 10 mM sodium phosphate (pH 6.5), and 8 mM $MgCl_2$ and injected into a column that was equilibrated with the same buffer. The linear reverse gradient was made with 10 mM sodium phosphate (pH 6.5) and 10% (w/w) methanol, and desorption from the column was monitored at 254 nm. According to this chromatograph, the tRNA purified by gel electrophoresis was more than 80% homogeneous. The HPLC procedure was also often used to purify tRNAs without the gel electrophoresis step.

*Labeling of tRNAs.* The tRNAs were labeled at the 5′ end with $^{32}$P according to standard procedures (Siberklang et al., 1977) with only minor modification. For 1 $\mu$g of tRNA, 0.5 unit of alkaline phosphatase (for dephosphorylation of the 5′ end) and 1 unit of polynucleotide kinase (for rephosphorylation with [$\gamma$-$^{32}$P]ATP) were used. The end-labeled tRNA was resolved by electrophoresis on a 12% polyacrylamide–7 M urea sequencing gel.

*Equilibrium Constant Determination.* The nitrocellulose filter binding methods originally developed by Yarus and Berg (1967a) were used to determine the equilibrium constant between the tRNAs and alanyl-tRNA synthetase. The fixed concentrations of the 5′-end-labeled tRNA were mixed with increasing concentrations of enzyme. The buffers used were 60 mM sodium acetate, 10 mM $MgCl_2$, and 2 mM DTT (pH 5.0) or 60 mM potassium phosphate, 10 mM $MgCl_2$, and 2 mM DTT (pH 5.5). The solution of the equilibrated tRNA–synthetase complex was filtered through a nitrocellulose disk (Schleicher & Schuell, BA85, 0.45 $\mu$m) that was prewet with the same buffer (in the absence of DTT) with a constant flow rate (about 1 mL/min) at room temperature (23 °C). The filter was then washed with the same buffer to remove free tRNA molecules (the volume of the washing buffer was 10 times that of the original sample volume). The filter-bound radiolabeled tRNA was counted in the presence of Hydroflour scintillation fluid.

The data were treated with a linear least-squares fit to an equation derived from the simple dissociation equilibrium constant ($K_d$)

$$K_d = [\text{tRNA}][\text{E}]/[\text{tRNA·E}]$$

where [tRNA·E] denotes the concentration of the complex at equilibrium. Because the concentrations of the enzyme used for each data point are in excess of the concentrations of tRNAs (from 2 to 6 orders of magnitude), the above equation can be simplified and linearized:

$$[\text{tRNA·E}]/([\text{tRNA}]_0 - [\text{tRNA·E}]) = (1/K_d)[\text{E}]_0$$

where $[\text{tRNA}]_0$ and $[\text{E}]_0$ indicate total tRNA and enzyme concentrations, respectively.

*Enzyme Assays.* The assay for the aminoacylation of $tRNA^{Ala}$ by alanyl-tRNA synthetase was adapted from the procedure described in Schreier and Schimmel (1972) with several modifications. The assay, at pH 5.5, was done in 60 mM potassium phosphate, 10 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, and 20 $\mu$M [$^3$H]alanine at 25 °C. At ph 7.5, bovine serum albumin (0.1 mg/mL) was included in the same buffer used for the reaction at pH 5.5, and the temperature was maintained at 37 °C. The alanine concentration used for the assays is subsaturating. [Because the $K_m$ for alanine is 240 $\mu$M (Hill & Schimmel, 1989), raising the alanine concentration to saturating levels requires impractical amounts of the radiolabeled substrate.] It was shown previously that the $K_m$ for $tRNA^{Ala}$ was not strongly dependent on the alanine concentration (Jasin et al., 1985).

The concentrations of tRNA and of alanyl-tRNA synthetase are given in each of the figure legends. For each time point in the assay, the background radioactivity was measured in the absence of tRNA and was subtracted from the radioactivity obtained in the aminoacylation reaction.

## Results

*Test for Inhibition of Aminoacylation by $A^3$·$U^{70}$ $tRNA^{Ala}$ at pH 7.5.* Figure 1a shows the sequence and cloverleaf structures of $tRNA^{Ala/UGC}$ and $tRNA^{Ala/CUA}$. The nine nucleotide differences between these two tRNAs are indicated. The $tRNA^{Ala/UGC}$ is a commercially available preparation that was purified further and has an amino acid acceptance of 1500 pmol per $A_{260}$. This tRNA species was utilized in part for reasons of convenience. The $tRNA^{Ala/CUA}$ is based on the sequence of $tRNA^{Ala/GGC}$ (Mims et al., 1985) with four substitutions in the anticodon loop (the anticodon trinucleotide and U38 → A) so as to create an efficient amber suppressor [see discussion and references in Hou and Schimmel (1988)]. These substitutions do not alter the identity as an alanine tRNA in vivo, and in preliminary studies the $tRNA^{Ala/UGC}$ and $tRNA^{Ala/CUA}$ species were equivalent, efficient substrates for alanyl-tRNA synthetase (Hou & Schimmel, 1988). The various $tRNA^{Ala/CUA}$ species are less readily obtained in large amounts because they are isolated by gel electrophoresis (see Materials and Methods). The $tRNA^{Ala/CUA}$ so isolated has

[1] Abbreviations: TBE buffer, 89 mM Tris–borate and 2 mM EDTA, pH 8.0; DTT, dithiothreitol.

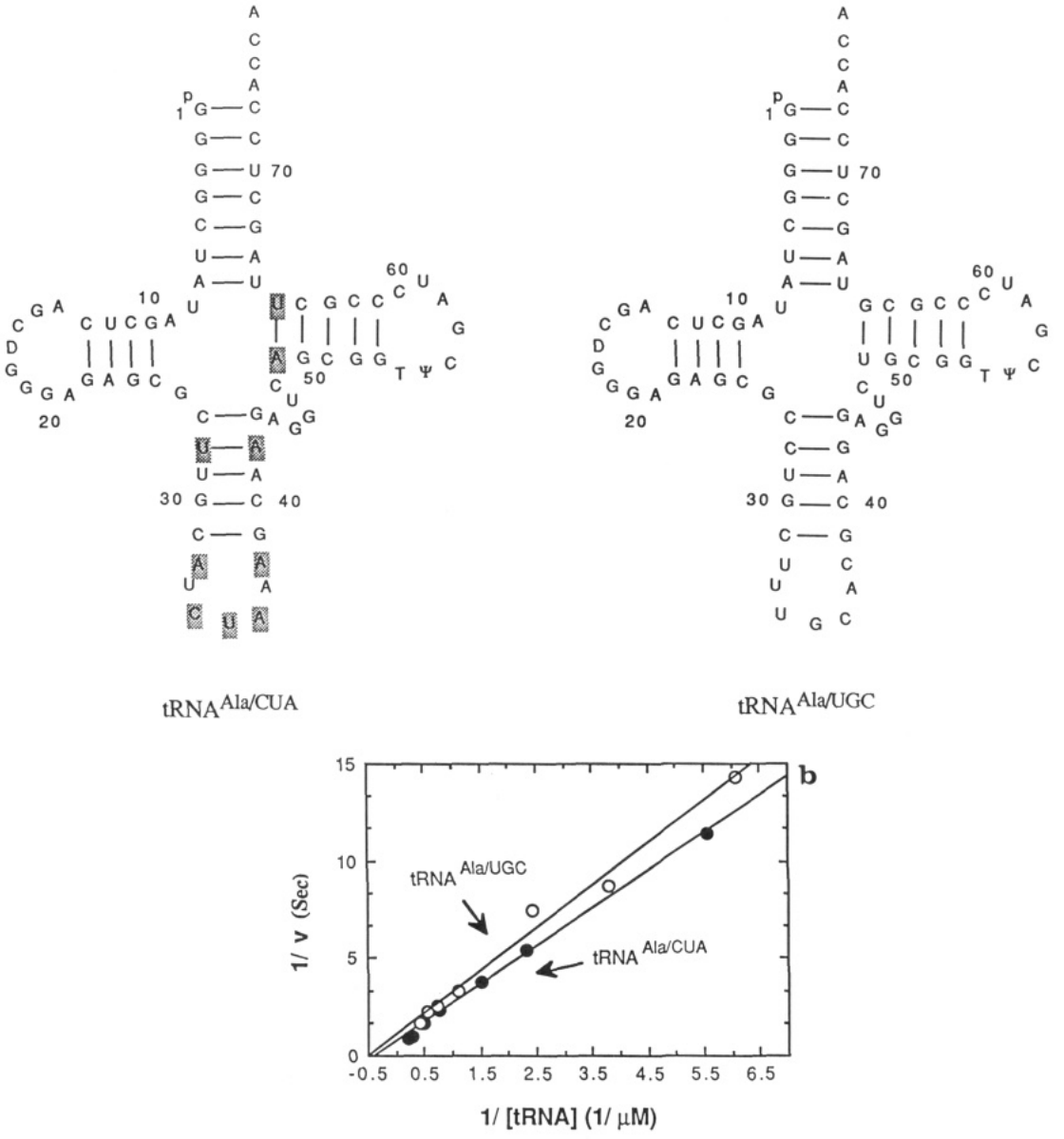


FIGURE 1: (a) Species of $tRNA^{Ala}$ that were used in the studies. The cloverleaf structures of $tRNA^{Ala/UGC}$ and $tRNA^{Ala/CUA}$ are shown. The shaded bases of $tRNA^{Ala/CUA}$ are the ones that are different from $tRNA^{Ala/UGC}$. (b) Lineweaver–Burk plot of initial rate of aminoacylation vs concentration of $tRNA^{Ala/UGC}$ and $tRNA^{Ala/CUA}$ at pH 7.5, 37 °C. The enzyme concentration was 10 nM. The open circles give results with $tRNA^{Ala/UGC}$, and the solid circles were obtained with $tRNA^{Ala/CUA}$. $K_m$ and $k_{cat}$ values of $tRNA^{Ala/UGC}$ and $tRNA^{Ala/CUA}$ are 2.2 μM (1.0 $s^{-1}$) and 2.9 μM (1.8 $s^{-1}$), respectively, where $k_{cat}$ is given in parentheses.

an amino acid acceptance equal to or greater than that of $tRNA^{Ala/UGC}$.

The kinetic behavior of the two tRNA substrates was investigated further. Figure 1b shows that $k_{cat} = 1.0\ s^{-1}$ and $K_m = 2.2\ \mu M$ for $tRNA^{Ala/UGC}$ and that the parameters are $1.8\ s^{-1}$ and 2.9 μM, respectively, for $tRNA^{Ala/CUA}$. (Note that the $k_{cat}$ values are determined at subsaturating concentrations of alanine, for reasons that are discussed under Materials and Methods.) The apparent second-order rate constant $k_{cat}/K_m$ is an overall measure of catalytic efficiency and is virtually identical for the two substrates ($4.5 \times 10^5\ M^{-1}\ s^{-1}$ for $tRNA^{Ala/UGC}$ and $6.2 \times 10^5\ M^{-1}\ s^{-1}$ for $tRNA^{Ala/CUA}$). This result is consistent with genetic studies and tRNA footprinting experiments which suggest that the anticodon loop does not interact with alanyl-tRNA synthetase (Hou & Schimmel, 1988; Park & Schimmel, 1988).

The $A^3 \cdot U^{70}$ or $G^3 \cdot C^{70}$ $tRNA^{Ala/CUA}$ fails to accept alanine by alanyl-tRNA synthetase (Hou & Schimmel, 1988). In the context of the Michaelis–Menten mechanism, this phenomenon can be explained as an effect on $K_m$ and/or on $k_{cat}$. The binding affinities of mutant tRNAs with the enzyme can be directly tested by determining the dissociation equilibrium constants ($K_d$). The nitrocellulose filter binding assay has been successfully used for the $K_d$ measurements of synthetase–tRNA complexes at low pH. However, because the binding efficiency of an aminoacyl-tRNA synthetase to nitrocellulose filters at pH 7.5 is very low (Yarus & Berg, 1970), $K_d$ measurements could not be attempted with this method. An indirect approach is to obtain the inhibition constant ($K_I$) of a mutant tRNA in the aminoacylation reaction of $tRNA^{Ala}$ by alanyl-tRNA synthetase. If a mutant $tRNA^{Ala}$ competes with wild-type $tRNA^{Ala}$ for the same site on the enzyme and thus acts as a competitive inhibitor, then the $K_I$ represents the dissociation constant for the mutant tRNA. For a systematic kinetic study, we chose $A^3 \cdot U^{70}$ $tRNA^{Ala/CUA}$ as the mutant species and $tRNA^{Ala/UGC}$ as the wild-type species.

At a fixed $tRNA^{Ala/UGC}$ concentration (below $K_m$), an increasing amount of $A^3 \cdot U^{70}$ mutant tRNA was added and the

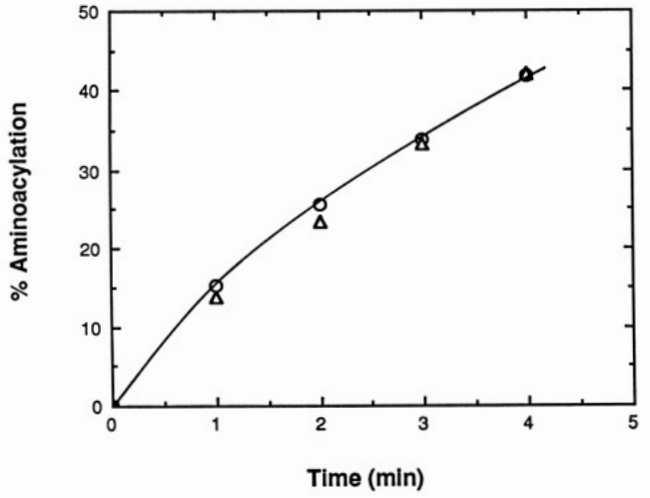


FIGURE 2: Lack of inhibition of aminoacylation at pH 7.5, 37 °C, by $A^3 \cdot U^{70}$ $tRNA^{Ala}$. The open circles indicate the aminoacylation of $tRNA^{Ala/UGC}$ (0.75 $\mu$M) with alanyl-tRNA synthetase (30 nM). The triangles were obtained under the same reaction conditions by the addition of 13 $\mu$M $A^3 \cdot U^{70}$ $tRNA^{Ala/CUA}$. The concentration of the mutant tRNA was determined by the absorbance at 260 nm. The percentage of aminoacylation was calculated from the plateau value of a control experiment.

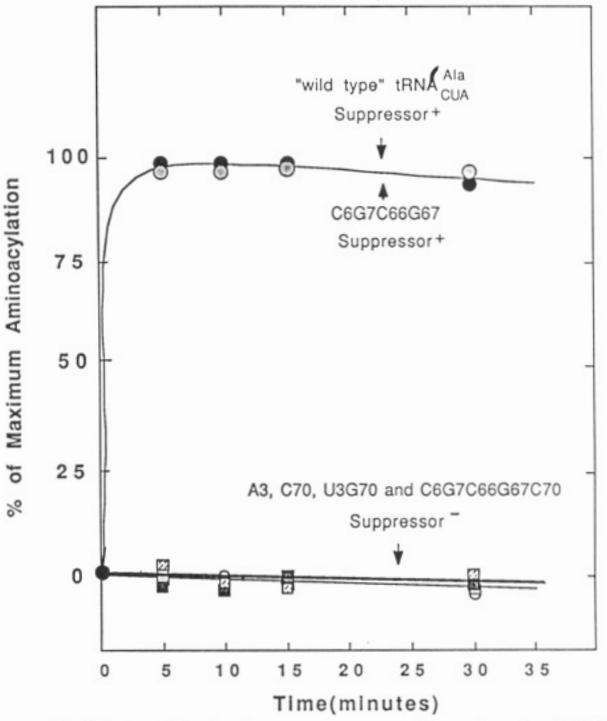


FIGURE 3: Substrate levels of enzyme cannot aminoacylate the $A^3 \cdot U^{70}$, $G^3 \cdot C^{70}$, or $U^3 \cdot G^{70}$ tRNA species. The concentration of each tRNA species was 4 $\mu$M, and each was incubated with 20 $\mu$M enzyme. The aminoacylation reaction was done at pH 7.5, 37 °C. The suppressor$^+$ or suppressor$^-$ designates which tRNA species are charged or not charged, respectively, with alanine in vivo (Hou & Schimmel, 1988).

effect on aminoacylation was observed. As seen in Figure 2, we did not observe significant inhibition of the aminoacylation of 0.75 $\mu$M $tRNA^{Ala/UGC}$ when up to 13 $\mu$M $A^3 \cdot U^{70}$ $tRNA^{Ala/CUA}$ was used. Assuming that the $A^3 \cdot U^{70}$ $tRNA^{Ala}$ binds to the enzyme, this observation enables us to estimate the lower bound for the inhibition constant, $K_I$. From the equation that describes competitive inhibition, we estimate that the $K_I$ of $A^3 \cdot U^{70}$ $tRNA^{Ala}$ is greater than 95 $\mu$M. (If $K_I$ is 95 $\mu$M, then there would be 10% of inhibition when $tRNA^{Ala/UGC}$ is at a concentration of 0.75 $\mu$M. This level of inhibition would have been detected in our assay, but repeated attempts to observe inhibition were unsuccessful.) We did not pursue the test for inhibition with higher than 13 $\mu$M $A^3 \cdot U^{70}$ $tRNA^{Ala/CUA}$ mutant species because a dissociation constant higher than 100 $\mu$M at pH 7.5 falls into the range of nonspecific interactions between noncognate tRNAs and aminoacyl-tRNA synthetases (Soll & Schimmel, 1974; Schimmel & Soll, 1979). [In some instances, the $K_d$ for the nonspecific interaction is only 1 or 2 orders of magnitude higher than that for the specific interaction (Ebel et al., 1973; Lam & Schimmel, 1975).] We did not attempt to measure the $K_d$ value of the mutant tRNA at pH 7.5 by using other physicochemical methods because we were not convinced that a $K_d$ higher than 100 $\mu$M is meaningful, and it is also difficult to obtain the large quantities of pure mutant tRNA that would be required. On the basis of the results shown in Figure 2, it can be concluded that a single $G^3$ to $A^3$ mutation weakens the binding with alanyl-tRNA synthetase at pH 7.5 by more than a factor of 30 ($K_I/K_m$ > 95 $\mu$M/2.9 $\mu$M). Thus, the lack of aminoacylation under these conditions results at least in part from the weaker binding of the mutant tRNA to the enzyme.

The lower binding affinity may not be the sole factor for the failure to aminoacylate the $A^3 \cdot U^{70}$ $tRNA^{Ala/CUA}$. The mutant tRNAs are overexpressed in vivo (Hou & Schimmel, 1988), which is easily observed from gel electrophoresis or from the HPLC profile of the crude total tRNA mixture. The HPLC used for the fractionation of the total tRNA can resolve most of the 20 tRNA species. The peak area of the overexpressed suppressor tRNA is at least 100-fold greater than that of the endogenous *E. coli* $tRNA^{Ala}$ species. This suggests that if $k_{cat}$ for the mutant tRNA is close to $k_{cat}$ for the wild-type tRNA, then the mutant tRNA could be charged with alanine in vivo, resulting in the suppressor$^+$ phenotype (Hou & Schimmel, 1988). Thus, the failure to aminoacylate mutant $tRNA^{Ala}$ may also be due to an effect on $k_{cat}$. The investigation of this possibility is described below.

*No Detection of Aminoacylation at pH 7.5 with Substrate Levels of Enzyme.* For the inhibition test shown in Figure 2, only catalytic amounts of enzyme were used. As shown in Figure 3, even with an enzyme concentration of 20 $\mu$M (5-fold higher than the tRNA concentration), the $A^3 \cdot U^{70}$, $G^3 \cdot C^{70}$, and $U^3 \cdot G^{70}$ variants of $tRNA^{Ala/CUA}$ were not aminoacylated. If the mutant tRNAs were charged but had an extremely slow "off" rate, then an initial burst of aminoacylation above the background level should be observed at this high enzyme concentration (Soll & Schimmel, 1974). However, no initial burst was observed. It should also be noted that a tRNA with multiple mutations in the acceptor stem at sites other than the $G^3 \cdot U^{70}$ base pair is efficiently aminoacylated (Figure 3).

*Further Investigations at pH 5.5.* As stated earlier, the nitrocellulose filter binding assay can be used to determine $K_d$ at lower pH values (Yarus & Berg, 1970). We chose pH 5.5 because, at this pH, the binding constant can be measured accurately and at the same time aminoacylation (albeit at a much lower rate than that at pH 7.5) can be observed. In general, complex formation between an aminoacyl-tRNA synthetase and its cognate tRNA is much stronger at lower compared to higher pH (Schimmel & Soll, 1979). This was demonstrated in the case of alanyl-tRNA synthetase and $tRNA^{Ala}$ by an RNase A protection assay (Park & Schimmel, 1988).

Figure 4 shows the binding curves of three tRNAs at pH 5.5. Alanyl-tRNA synthetase is a tetramer, and each subunit of the enzyme binds one molecule of $tRNA^{Ala}$ (Regan et al., 1987). As observed by Regan et al. (1987), the binding of neither wild-type nor mutant tRNA to the enzyme shows

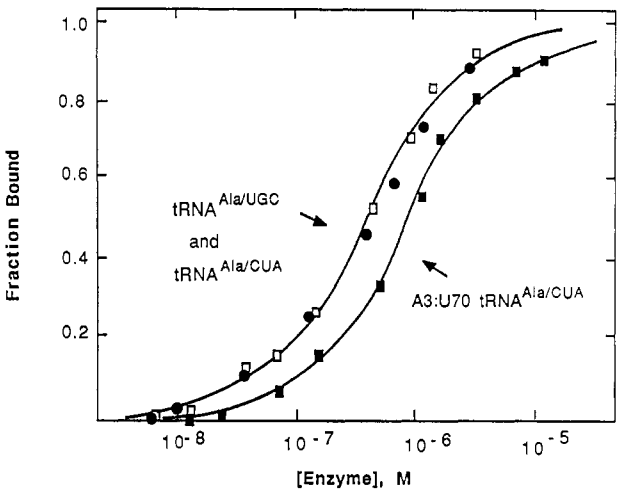


FIGURE 4: Binding of wild-type and mutant alanine tRNA species at pH 5.5, 23 °C. The fraction of tRNA bound onto a nitrocellulose filter is plotted against increasing concentrations of alanyl-tRNA synthetase that are given by a logarithmic scale. The dissociation constants were determined from the slopes of linear least-squares plots after the data points were converted into the linear form that is described under Materials and Methods.

Table I: Dissociation Constants for Complexes of Alanyl-tRNA Synthetase with Wild-Type and Mutant Alanine tRNAs

| tRNA species | $K_d$[a] | $K_m$ or $K_I$[b] ($\mu$M) |
|---|---|---|
| | (a) pH 5.5, 23 °C | |
| Ala/UGC | 0.28 $\mu$M | 0.22 ($K_m$) |
| Ala/CUA | 0.42 $\mu$M | nd[c] |
| $A^3 \cdot U^{70}$ Ala/CUA | 1.2 $\mu$M | 1.5 ($K_I$) |
| $G^3 \cdot C^{70}$ Ala/CUA | 1.4 $\mu$M | nd |
| | (b) pH 5.0, 23 °C | |
| Ala/UGC | 50 nM | |
| Ala/CUA | 32 nM | |
| $A^3 \cdot U^{70}$ Ala/CUA | 52 nM | |
| $G^3 \cdot C^{70}$ Ala/CUA | 45 nM | |

[a] Determined by the nitrocellulose filter assay. The error for $K_d$ is in the range of 50%. [b] Determined by kinetic measurement. [c] Not determined.

cooperativity. The data points of the $tRNA^{Ala/UGC}$ and of the wild-type suppressor $tRNA^{Ala/CUA}$ are virtually superimposed within experimental error and give $K_d$ values of 280 and 420 nM, respectively (Table Ia). These two tRNAs have nine nucleotide sequence differences and eight of these are located in the anticodon stem and loop. However, the acceptor stems of the two tRNAs have the same nucleotide sequence. According to RNase footprinting of the enzyme–tRNA complex (Park & Schimmel, 1988) and genetic studies (Hou & Schimmel, 1988), the anticodon loop of the tRNA molecules is not critical for the tRNA–synthetase interaction in this system.

As shown in Figure 4 and Table Ia, the binding at pH 5.5 of $A^3 \cdot U^{70}$ and $G^3 \cdot C^{70}$ $tRNA^{Ala}$ to the enzyme is only 3–5-fold weaker than the binding of the wild-type $G^3 \cdot U^{70}$ $tRNA^{Ala}$ Aware that the nitrocellulose filter binding assay may not discriminate productive from nonproductive enzyme–tRNA complex formation, the test for inhibition was also done to verify the $K_d$ of the mutant tRNA. Under the same conditions used for the filter binding assay (at pH 5.5), $K_m$ of $tRNA^{Ala/UGC}$ was determined to be 0.22 $\mu$M, which agrees well with the $K_d$ measured by the filter binding assay (Table Ia).

Figure 5 shows that increasing concentrations of the $A^3 \cdot U^{70}$ mutant tRNA inhibit the aminoacylation of $tRNA^{Ala/UGC}$ by alanyl-tRNA synthetase, which is in contrast to the result obtained at pH 7.5 (Figure 2). When the incubation time is

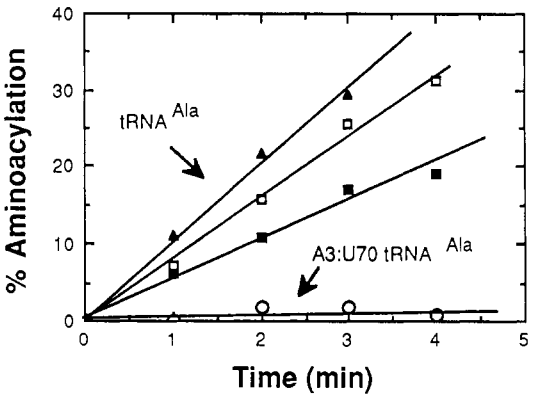


FIGURE 5: Inhibition of aminoacylation at pH 5.5, 23 °C, by $A^3 \cdot U^{70}$ $tRNA^{Ala}$. The solid triangles give results for the initial phase of an aminoacylation reaction with 0.2 $\mu$M $tRNA^{Ala/UGC}$, and the open circles were from the reaction with 1.8 $\mu$M $A^3 \cdot U^{70}$ $tRNA^{Ala/CUA}$. For inhibition measurements, 0.71 $\mu$M (open squares) and 1.8 $\mu$M (filled squares) $A^3 \cdot U^{70}$ mutant tRNAs were added to 0.2 $\mu$M $tRNA^{Ala/UGC}$. The enzyme concentration was 40 nM. The plateau value for aminoacylation of $tRNA^{Ala/UGC}$ was obtained independently to scale the ordinate values.

extended further, the plots for aminoacylation of $tRNA^{Ala/UGC}$ (with and without the mutant tRNA) reach the same plateau; when higher concentrations (16-fold higher than $K_m$) of the wild-type $tRNA^{Ala}$ were used, the inhibition was overcome (data not shown). These phenomena indicate that the inhibition by the mutant tRNA is competitive. On the basis of the competitive inhibition equation, the $K_I$ calculated from Figure 5 is 1.5 $\mu$M, which is in good agreement with the $K_d$ observed by the filter binding assay (Table Ia). At pH 5.5, the $A^3 \cdot U^{70}$ mutant tRNA does not charge with alanine even though the binding to enzyme is almost as strong as that of the wild-type $tRNA^{Ala}$ (Figure 4). Therefore, under these conditions, alanyl-tRNA synthetase discriminates against the $A^3 \cdot U^{70}$ $tRNA^{Ala/CUA}$ species by a sharp reduction in $k_{cat}$.

We also measured the dissociation constant of $tRNA^{fMet}$ with alanyl-tRNA synthetase as an example of a noncognate interaction. The $K_d$ at pH 5.5 was 3.7 $\mu$M. This value is only 3-fold higher than the values obtained with the mutant $tRNA^{Ala}$s (Table Ia). A high concentration of $tRNA^{fMet}$ (25 $\mu$M), however, did not inhibit the aminoacylation of $tRNA^{Ala}$ by alanyl-tRNA synthetase under the same conditions as those used in Figure 5. (The binding and inhibition data with $tRNA^{fMet}$ are not shown.) This suggests that $tRNA^{fMet}$ interacts with alanyl-tRNA synthetase at a different site and that the filter binding assay does not discriminate this from binding to the catalytic site. Because inhibition could be observed with the $A^3 \cdot U^{70}$ $tRNA^{Ala}$ mutant, and because $K_d$ and $K_I$ values are in agreement, the interaction between the mutant $tRNA^{Ala}$ and the enzyme is likely to be similar to that of the wild-type $tRNA^{Ala}$ with the enzyme.

The test for inhibition conducted at pH 5.5 and 7.5 demonstrates that the complex of mutant $tRNA^{Ala}$ with alanyl-tRNA synthetase is more pH sensitive than is the complex with wild-type $tRNA^{Ala}$. From pH 5.5 to pH 7.5, $K_d$ for wild-type $tRNA^{Ala}$ increases by a factor of 10 (if $K_d$ is close to $K_m$). However, in the case of the $A^3 \cdot U^{70}$ $tRNA^{Ala}$, it increases by more than a factor of 80. To explore further this question, additional binding measurements were done at pH 5.0. At this pH, the wild-type and the mutant $tRNA^{Ala}$s have almost the same dissociation constants (Table Ib). Between pH 5.0 and pH 5.5, however, $K_d$ increases by a factor of 6–10 in the case of the wild-type $tRNA^{Ala/UGC}$ and $tRNA^{Ala/CUA}$, but by a factor of 20 for the mutant tRNAs. We attempted to determine $K_d$ values at pH 6.0 in order to investigate further

these pH effects. However, the binding efficiency at pH 6.0 of the enzyme to the filter was too low to obtain valid experimental results.

## Discussion

Earlier work has suggested a role for the $k_{cat}$ parameter in the discrimination of cognate from noncognate tRNAs (Ebel et al., 1973; Roe et al., 1973; Bare & Uhlenbeck, 1985; Schulman & Pelka, 1985). Studies of synthetase–tRNA complexes by fast kinetic methods have suggested that a conformational change occurs in the complex of yeast Phe-tRNA synthetase with yeast $tRNA^{Phe}$. When the noncognate *E. coli* $tRNA^{Tyr}$ (which is not aminoacylated) was bound to the same enzyme, the unimolecular conformational change was not observed (Rigler et al., 1976). These findings suggested that a specific kinetic intermediate could not form in the noncognate complex. Alternatively, the noncognate tRNA may have bound to a site on the enzyme different from that of the cognate tRNA (Rigler et al., 1976).

Our data at pH 7.5 establish that the $A^3 \cdot U^{70}$ $tRNA^{Ala}$ binds significantly less efficiently (if at all) to the enzyme than does wild-type $tRNA^{Ala}$. Because substrate levels of enzyme fail to aminoacylate four different mutant tRNAs under these conditions (Figure 3), it is likely that the $k_{cat}$ parameter is affected as well. Under at least some conditions, the release of the charged tRNA from the enzyme is judged to be rate determining in the overall aminoacylation reaction (Yarus & Berg, 1969; Eldred & Schimmel, 1972; Soll & Schimmel, 1974). The experiments with substrate levels of enzyme rule out the possibility that the failure to observe aminoacylation is due to an extraordinarily slow release of charged mutant species, however. This is because, with high enzyme concentrations, sufficient aminoacylated tRNA would be produced in the first cycle of the reaction so as to be detectable by our assay system. Thus, a reduction in $k_{cat}$ must occur at a step that precedes the stable attachment of amino acid to tRNA.

At pH 5.5, the complexes of the $A^3 \cdot U^{70}$ and $G^3 \cdot C^{70}$ $tRNA^{Ala}$ species with enzyme are within 1 kcal/mol in stability of that of the wild-type $tRNA^{Ala}$–enzyme complex. Thus, the differences in relative affinities observed at pH 7.5 are overcome by the reduced pH. There is a pH-dependent conformational change in some synthetases (with an apparent approximate p$K$ of 6.0) that is correlated with an elevation in affinity for tRNA [cf. Lam and Schimmel (1975)]. To determine whether alanyl-tRNA synthetase has a similar pH-dependent conformational change, we studied its fluorescence emission. As shown in Figure 6, the emission is sensitive to pH with an approximate p$K$ of 6 suggested by the fluorescence titration.

The increase in affinity for tRNA at lower pH values is also accompanied by a drop in the rate of the overall aminoacylation reaction, which may indicate that the release of the charged tRNA is the rate-determining step in aminoacylation of the wild-type tRNA under these conditions. The reduced pH apparently introduces new enzyme–tRNA contacts that strengthen the complex and lessen the discrimination at the level of binding. Interestingly, this reduction at the level of binding is not accompanied by an ability to aminoacylate the $A^3 \cdot U^{70}$ $tRNA^{Ala}$ species (Figure 5). This may indicate that at pH 5.5 one or more sites on the enzyme that provide discrimination at the level of $k_{cat}$ are distinct from those that are used for binding.

According to footprinting experiments of the $tRNA^{Ala}$–enzyme complex (Park & Schimmel, 1988), the enzyme protects (from ribonuclease attack) portions of the anticodon stem and D-loop of $tRNA^{Ala}$ in addition to giving extensive protection in parts of the acceptor stem. Some of the nucleotides that are protected can be varied without changing the ability of $tRNA^{Ala}$ to be aminoacylated with alanine in vivo (Hou & Schimmel, 1988). Therefore, these sites are not essential for the identity of $tRNA^{Ala}$. If the mutations at these positions affect binding to the enzyme (i.e., a $K_m$ effect), then these mutants may be aminoacylated with alanine (and thus give the suppressor$^+$ phenotype), because of being overexpressed in vivo. On the other hand, substitutions at the 3 and 70 positions are defective for aminoacylation in vivo even with overproduction of the mutant tRNAs. For these mutants, $k_{cat}/K_m$ for aminoacylation with alanine as determined in vitro appears to be reduced by more than enough to compensate for overproduction of mutant tRNAs in vivo (cf. Figures 2 and 3).

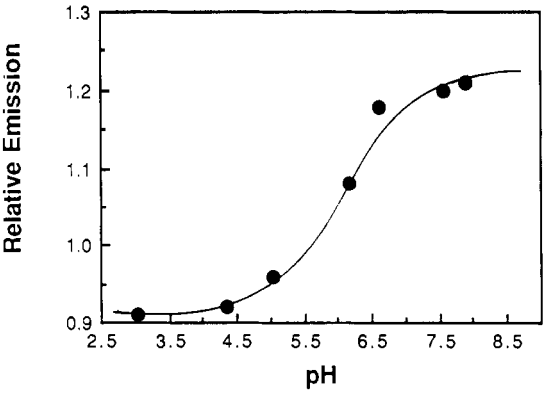


FIGURE 6: pH-dependent relative fluorescence emission at 335 nm of 1 $\mu$M alanyl-tRNA synthetase at 23 °C. The fluorescent emission spectra (SPEX Model DM 1B) were observed at a wavelength of 335 nm with a 290-nm excitation wavelength at a 6-nm slit width. The buffer was 1.5 mM sodium citrate–borate–phosphate and 0.15 M NaCl, and the pH of the buffer (in the absence of enzyme) was adjusted with HCl. For each spectrum, the base line was subtracted. The reversibility of the fluorescence change was demonstrated. The emission changes are mainly from the change of environment of tryptophan residues in the enzyme. On the basis of these results, we can expect that as pH increases, tryptophan residues are less exposed to the solvent.

McClain et al. (1988) report that an amber codon of the gene for dihydrofolate reductase may be suppressed with $A^3 \cdot U^{70}$ and $U^3 \cdot G^{70}$ $tRNA^{Ala}$ mutant suppressor species and that alanine is among the amino acids that are inserted by the misacylated mutant tRNA. On the basis of this and other results they suggest that alanine acceptor identity is associated with a helix irregularity. However, the $A^3 \cdot U^{70}$ and $U^3 \cdot G^{70}$ mutant species do not suppress (or at best poorly suppress) the alanine-requiring *trpA(UAG234)* amber allele (Hou & Schimmel, 1988; McClain et al., 1988). Moreover, these mutants cannot be aminoacylated in vitro with substrate levels of enzyme (Figure 3). Thus, the amber suppressor assay (at least with respect to the protein produced by suppression of a dihydrofolate reductase amber codon) can be relatively insensitive to major changes in the kinetic parameters for molecular recognition. (Possibly a residual aminoacylation activity of these mutants in vivo is sufficient to give minor amounts of charged mutant $tRNA^{Ala}$ that are sequestered in vivo by elongation factor Tu and the ribosomes.) Because mutants that do not suppress or at best poorly suppress the *TrpA(UAG234)* amber allele are also defective for aminoacylation in vitro with alanine [Hou and Schimmel (1988) and see above], the suppression phenotype of this amber allele may be more sensitive to changes in the parameters for the enzyme–tRNA interaction.

Alanyl-tRNA synthetase is a tetramer of identical polypeptides of 875 amino acids (Putney et al., 1981). There is

a domain for adenylate synthesis that is located in the amino-terminal 368 amino acids (Jasin et al., 1983). Within this domain, there is evidence that Lys-73 is proximal to the 3′ end of the bound tRNA where it can react with the aminoacyl adenylate (Hill & Schimmel, 1989). Crystals of this domain that are suitable for diffraction analysis have been obtained and will yield structural information on this part of the enzyme (Frederick et al., 1988). Determinants for binding to parts other than the 3′ end of the tRNA are located on the carboxy-terminal side of Arg-368, however. In particular, the 93 amino acids from Arg-368 to Asp-461 have been shown to be especially important for the enzyme–tRNA binding interaction and for catalysis of the reaction of the alanyl adenylate with $tRNA^{Ala}$ (Regan et al., 1987). Therefore, it is plausible that this segment of the structure distinguishes, by interactions that determine $k_{cat}$ and $K_m$ parameters, the G·U base pair from others at positions 3 and 70.

Because the presence of the $G^3 \cdot U^{70}$ base pair is sufficient to enable tRNA species of widely different sequences to be aminoacylated with alanine, and because in *E. coli* the G·U pair is unique to $tRNA^{Ala}$, the enzyme had to evolve a rigorous system to distinguish $tRNA^{Ala}$ from those tRNAs that differ by only a single nucleotide at this position. Thus, this system prevents the enzyme from reacting with tRNAs that have a $G^3 \cdot C^{70}$ base pair (such as those for glutamine, glycine, histidine, leucine, lysine, tryptophan, and valine) and an $A^3 \cdot U^{70}$ base pair (specific isoacceptors of arginine and serine tRNAs) (Sprinzl et al., 1987). The data presented here suggest that both catalytic and binding determinants on the enzyme are utilized at pH 7.5 for this discrimination and thereby provide a double barrier against aminoacylation of the incorrect tRNA. Moreover, a failure to bind strongly at pH 7.5 those tRNA species that differ by only one nucleotide at the 3·70 position, as is suggested by the data in Figure 2 on $A^3 \cdot U^{70}$ $tRNA^{Ala}$, would prevent them not only from being substrates but also from being inhibitors of the enzyme.

## Acknowledgments

We thank Dr. Christopher Francklyn and Dr. Kelvin Hill for helpful comments on the manuscript.

## References

Bare, L., & Uhlenbeck, O. C. (1985) *Biochemistry 24*, 2354–2360.

Ebel, J.-P., Giege, R., Bonnet, J., Kern, D., Befort, N., Bollack, C., Fasiolo, F., Gangloff, J., & Dirheimer, G. (1973) *Biochimie 55*, 547–557.

Eldred, E. W., & Schimmel, P. R. (1972) *Biochemistry 11*, 17–23.

Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, B. R., Koch, G. L. E., & Hartley, B. S. (1975) *Biochemistry 14*, 1–4.

Franckyn, C., & Schimmel, P. (1989) *Nature* (in press).

Frederick, C. A., Wang, A. H.-J., Rich, A., Regan, L., & Schimmel, P. (1988) *J. Mol. Biol. 203*, 521–522.

Hill, K., & Schimmel, P. (1989) *Biochemistry 28*, 2577–2586.

Hou, Y. M., & Schimmel, P. (1988) *Nature 333*, 140–145.

Jasin, M., Regan, L., & Schimmel, P. (1983) *Nature 306*, 441–447.

Jasin, M., Regan, L., & Schimmel, P. (1985) *J. Biol. Chem. 260*, 2226–2230.

Lam, S. S. M., & Schimmel, P. R. (1975) *Biochemistry 14*, 2275–2280.

McClain, W. H., & Foss, K. (1988) *Science 240*, 793–796.

McClain, W. H., Chen, Y.-M., Foss, K., & Schneider, J. (1988) *Science 242*, 1681–1684.

Mims, B. H., Pratter, N. E., & Murgola, E. J. (1985) *J. Bacteriol. 162*, 837–839.

Muramatsu, T., Nishikawa, K., Nemoto, F., Kuchino, Y., Nishimura, S., Miyazawa, T., & Yokoyama, S. (1988) *Nature 336*, 179–181.

Normanly, J., Ogden, R. C., Horvath, S. J., & Abelson, J. (1986) *Nature 321*, 213–219.

Pabo, P. O., & Sauer, R. T. (1984) *Annu. Rev. Biochem. 53*, 293–321.

Park, S. J., & Schimmel, P. (1988) *J. Biol. Chem. 263*, 16527–16530.

Putney, S. D., Royal, N. J., de Vegvar, H. N., Herlihy, W. C., Biemann, K., & Schimmel, P. (1981) *Science 213*, 1497–1501.

Regan, L. (1986) Ph.D. Thesis, Massachusetts Institute of Technology.

Regan, L., Bowie, J., & Schimmel, P. (1987) *Science 235*, 1651–1653.

Rigler, R., Pachmann, U., Hirsch, R., & Zachau, H. G. (1976) *Eur. J. Biochem. 65*, 307–315.

Roe, B., Sirover, M., & Dudock, B. (1973) *Biochemistry 12*, 4146–4154.

Rogers, M. J., & Soll, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6627–6631.

Sampson, J. K., DiRenzo, A., Behlen, L., & Uhlenbeck, O. C. (1988) *Science* (in press).

Schimmel, P. R., & Soll, D. (1979) *Annu. Rev. Biochem. 48*, 601–648.

Schreier, A. A., & Schimmel, P. R. (1972) *Biochemistry 11*, 1582–1589.

Schulman, L. H., & Pelka, H. (1985) *Biochemistry 24*, 7309–7314.

Schulman, L. H., & Abelson, J. (1988) *Science 240*, 1591–1592.

Schulman, L. H., & Pelka, H. (1988) *Science 242*, 765–768.

Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1977) *Nucleic Acids Res. 4*, 4091–4108.

Soll, D., & Schimmel, P. (1974) *Enzymes 10*, 489–538.

Sprinzl, M., Hartman, T., Meissner, F., Moll, H., & Vorderwulbecke, T. (1987) *Nucleic Acids Res. 15*, r53–r188.

Wurst, R. M., Vornakis, J. N., & Maxam, A. M. (1978) *Biochemistry 17*, 4493–4499.

Yarus, M., & Berg, P. (1967) *J. Mol. Biol. 28*, 479–490.

Yarus, M., & Berg, P. (1969) *J. Mol. Biol. 42*, 171–189.

Yarus, M., & Berg, P. (1970) *Anal. Biochem. 35*, 450–465.

Zhang, S.-B., Bronskill, P. M., Wang, Q.-S., & Wong, J. T. (1986) *J. Chromatogr. 360*, 282–287.