Codon-Dependent Rearrangement of the Three-Dimensional Structure of Phenylalanine tRNA, Exposing the T-ψ-C-G Sequence for Binding to the 50S Ribosomal Subunit†

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ABSTRACT: Codon–anticodon interaction induces an allosteric rearrangement of the three-dimensional structure of Phe-tRNA^{Phe} that exposes the T-ψ-C-G sequence for binding to the C-G-A-A sequence of the 5S rRNA within the 50S ribosomal subunit. The conformational change in the tRNA^{Phe} structure was followed by the binding of C-G-[^{3}H]A-[^{3}H]A to the T-ψ-C-G sequence, as measured by equilibrium dialysis at 10 mM Mg^{2+}. C-G-A-A (14 pmol) was bound to tRNA^{Phe} in the complete system containing elongation factor T_u·GTP·Phe-tRNA·(uridylyl-3′,5′)$_7$-uridine·30S ribosomes (100 pmol). At a Mg^{2+} concentration lower than 5 mM the rearrangement was dependent on elongation factor-T_u, whereas GTP could be replaced by guanylyl imidodiphosphonate. In the absence of elongation factor-T_u·GTP a sigmoidal C-G-A-A binding curve with respect to Mg^{2+} concentration was obtained, showing half-saturation at 6 mM Mg^{2+}. To achieve the change in the tRNA^{Phe} structure in the absence of 30S ribosomes, a twofold higher concentration of (uridylyl-3′,5′)$_7$-uridine had to be used. A sigmoidal curve was obtained again when the Mg^{2+} dependence of the C-G-A-A binding was followed, with 12 pmol of C-G-A-A being bound to 200 pmol of Phe-tRNA. Since T-ψ-C-G exposure should influence the binding of Phe-tRNA to 70S ribosomes, Phe-tRNA binding to 70S ribosomes was examined. In the "nonenzymatic" binding (i.e., no elongation factor-T_u·GTP) of Phe-tRNA a sigmoidal Mg^{2+} dependence was found, whereas the "enzymatic" binding (elongation factor-T_u·GTP present) showed a hyperbolic curve. With 30S ribosomes as controls, only hyperbolic binding curves were found. The Mg^{2+} dependence of AA-tRNA binding thus reflects the rearrangement of the tRNA structure.

One common feature of nearly all tRNAs whose sequences are known is the occurrence of the pentanucleotide G-T-ψ-C-G at a defined position within the tRNA structure (Zamir et al., 1965; Chirikdjian and Davis, 1970). Since the absence of this sequence has been confined, with a few exceptions, to the initiator tRNAs (Simsek et al., 1973; Petrissant, 1973; Piper and Clark, 1973), it is now generally accepted that this sequence plays an important role in ribosome–tRNA interaction for tRNAs participating in the peptide elongation cycle. This assumption was substantiated by the finding that the 5S rRNA of the 50S subunit contains a complementary sequence C-G-A-A at positions 43–46 (Brownlee et al., 1967). Its capacity to interact with the tRNA is most likely, because it appears to be in an exposed region of the 5S rRNA as judged by ribonuclease-T1 hydrolysis (Jordan, 1971) and N-oxidation of the adenosines (Erdmann et al., 1973a). However, the guanosine in position 44 is not susceptible to kethoxal modification (Noller and Herr, 1974; Delihas et al., 1975). It was hypothesized by Jordan (1971) that this sequence in the 5S rRNA binds the tRNA to the 50S ribosomal subunit by double-strand formation between the two sequences in question. This idea gained experimental support by the finding that T-ψ-C-G inhibits AA-tRNA binding to 70S ribosomes (Ofengand and Henes, 1969). Binding of initiator tRNA was not inhibited (Grummt et al., 1974). Although there is strong evidence from the experiments mentioned that 5S rRNA interacts with T-ψ-C-G, the data do not exclude that binding to 23S rRNA might also occur.

These results are explicable if one assumes that the oligonucleotide sequences in both tRNA and 5S rRNA are exposed. However, this is an oversimplification, because the inaccessibility of the T-ψ-C-G sequence in free tRNA has been shown by chemical modification, enzymatic digestion (Gauss et al., 1971), and complementary oligonucleotide binding (Pongs et al., 1973). Additional evidence for the inaccessibility of this sequence in the tRNA came from three-dimensional structure determinations of yeast tRNA^{Phe} (Kim et al., 1973; Robertus et al., 1974), which showed the T-ψ-C-G loop to be hydrogen bonded to the dihydrouridine loop.

To resolve this discrepancy between the obvious T-ψ-C-G 50S ribosome interaction and the inaccessibility of this sequence in either dissolved or crystallized tRNA, we proposed a more detailed model (Schwarz et al., 1974). In this model, we assume that the T-ψ-C-G sequence is buried in free tRNA and in the EF-T_u·GTP·AA-tRNA complex.[1] Recognition of an AA-tRNA by its codon then induces a change in the structure of the tRNA, as a result of which the T-ψ-C-G sequence becomes exposed for binding to the 50S subunit (Figure 1).

In this paper, we present experimental evidence that the capacity of T-ψ-C-G in tRNA^{Phe} to bind to the 50S subunit is controlled by codon–anticodon interaction. Codon–anticodon complex formation and binding of the complementary oligonucleotide C-G-A-A parallel each other when studied as a function of EF-T_u presence and Mg^{2+} concentration.

Experimental Procedure

Materials. Alkaline phosphatase (EC 3.1.3.1), pyruvate

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[1] Abbreviations used are: EF-T_u, elongation factor T_u; EF-T_s, elongation factor T_s; GMP-P(NH)P, guanylyl imidodiphosphonate; (U)$_8$, (uridylyl-3′,5′)$_7$-uridine; "nonenzymatic" Phe-tRNA binding, coded binding to ribosomes in the absence of EF-T_u·GTP; "enzymatic" Phe-tRNA binding, coded binding of the complex EF-T_u·GTP·Phe-tRNA to ribosomes; Phe-tRNA, phenylalanyl-tRNA^{Phe}; DEAE, diethylaminoethyl.

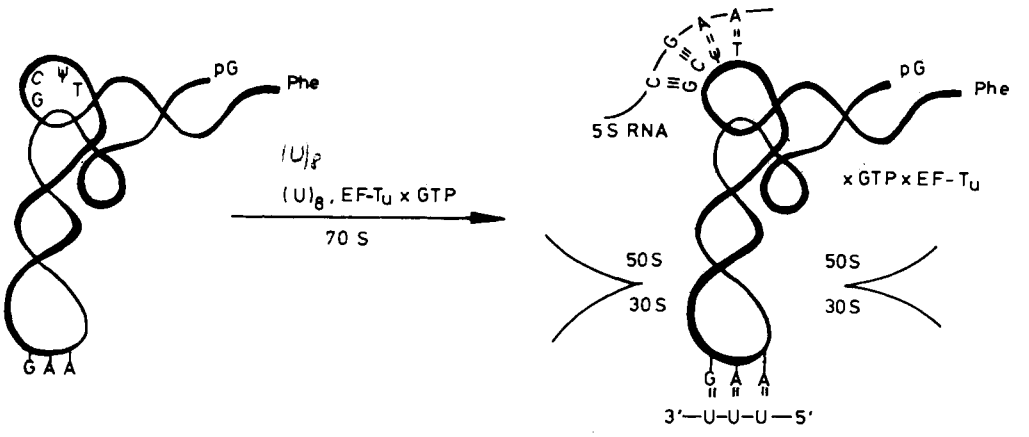

FIGURE 1: Schematic drawing of the proposed model. The mRNA-induced change of the conformation of the Phe-tRNA enables the Phe-tRNA to bind to the 5S rRNA of the 50S ribosomal subunit via the now exposed T-ψ-C-G sequence.

kinase (EC 2.7.1.40), snake venom phosphodiesterase (EC 3.1.4.1), ribonuclease T_1 (EC 3.1.4.8), *E. coli* MRE 600 tRNA, *E. coli* MRE 600 $tRNA^{Phe}$, and guanylyl imidodiphosphonate were purchased from Boehringer, Mannheim. C-G and all other nucleoside 5'-di- and triphosphates were supplied by Pharma Waldhof, Düsseldorf. [^{3}H]Adenosine 5'-diphosphate (sp act. 18 000 Ci/mol), [^{3}H]guanosine 5'-triphosphate (sp act. 17 000 Ci/mol), and [^{3}H]phenylalanine (sp act. 5 000 Ci/mol) were obtained from Amersham-Buchler, Braunschweig. *Escherichia coli* MRE 600 and *Micrococcus luteus* were received from Merck, Darmstadt. Dialysis membranes—readily permeable to nucleotides—came from Iris 3069, Rhône-Poulenc C., Paris.

Methods. 70S Ribosomes were isolated from mid-log-phase *E. coli* MRE 600 according to the method of Noll et al. (1973). 50S and 30S ribosomal subunits were prepared by zonal centrifugation (Leifer and Kreuzer, 1971). The ribosomal subunits were dissolved in 50 mM Tris-Cl, pH 7.5, 20 mM $Mg(OAc)_2$, 200 mM NH_4Cl, and 2 mM 1,4-dithioerythritol to a final concentration of 160 A_{260} units/ml. They were stored at −80 °C. Prior to use, appropriate aliquots of subunits were incubated for 25 min at 40 °C in storage buffer. This incubation was necessary to activate the ribosomal subunits (Zamir et al., 1971). Contamination of ribosomes by nucleases was assayed by incubation with [^{3}H]poly(U) at 4 °C as described by Voigt and Matthaei (1968). At this temperature no degradation of the polynucleotide was observed. *E. coli* polypeptide elongation factor T_u ($EF\text{-}T_u$) was prepared according to the method of Arai et al. (1972).

In order to obtain a completely nuclease-free elongation factor, $EF\text{-}T_u$ was recrystallized three times in the form of the binary complex $EF\text{-}T_u$·GDP. The final product showed only one band (20 μg of protein/gel) in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The crystalline $EF\text{-}T_u$·GDP complex was dissolved in 20 mM Tris-Cl, pH 7.5, 20 mM magnesium acetate, 2 mM 1,4-dithioerythritol, and 250 mM sucrose to a final concentration of 4 mg of $EF\text{-}T_u$·GDP/ml and stored at −80 °C. In the presence of stoichiometric amounts of $EF\text{-}T_s$, $EF\text{-}T_u$·GDP binds 1 mol of GTP/45 000 g of protein. Because $EF\text{-}T_u$·GTP was used for $tRNA^{Phe}$ binding assays, this complex was formed from $EF\text{-}T_u$·GDP by addition of a 2500-fold excess of GTP.

[2] Definition: an A_{260} unit is the quantity of material contained in 1.0 ml of a solution that has an absorbance of 1 at 260 nm when measured in a cell with 1-cm path length.

Ternary complex formation was assayed for by the method of Ravel et al. (1968).

E. coli tRNA was charged with [^{3}H]phenylalanine (sp act. 1000 Ci/mol) using purified synthetase preparation, as described by Traub et al. (1971). In the analytical tests, the charging of $tRNA^{Phe}$ was 60%, while on a preparative scale under the same conditions, it was 25%.

C-G-[^{3}H]A-[^{3}H]A was prepared from C-G and [^{3}H]adenosine 5'-diphosphate (sp act. 160 Ci/mol) with primer dependent polynucleotide phosphorylase (EC 2.7.7.8) isolated from *Micrococcus luteus* (Schetters et al., 1972). The yield of C-G[^{3}H]A-[^{3}H]A was 8% with respect to C-G. The oligonucleotide sequence and its specific activity (320 Ci/mol) were determined by use of a nucleoside analyzer (Gassen and Leifer, 1970). Octauridylate, $(U)_8$, was prepared by the same procedure as described above, using U-U as primer. The oligonucleotides produced were separated by DEAE-cellulose column chromatography. The peak containing the octanucleotide was purified as reported (Schetters et al., 1972).

Equilibrium dialysis was performed at 0–4 °C, as originally described by Uhlenbeck et al. (1970). Experimental details were presented in a recently published paper (Schwarz et al., 1974). Radioactivity was determined by withdrawing the contents of each compartment and counting four 20-μl aliquots in Bray's solution. The counting efficiency was 30%.

The polyuridylate-directed binding of [^{3}H]Phe-tRNA and $EF\text{-}T_u$·GTP·[^{3}H]Phe-tRNA to 70S and 30S ribosomes was measured by the nitrocellulose filter assay, according to the method of Nirenberg and Leder (1964), under conditions of optimal ribosomal activity (Kaufmann and Zamir, 1972). For equilibrium dialysis, as well as for ribosomal binding assays, the following buffer was used: 200 mM Tris-Cl, pH 7.5, 200 mM NH_4Cl, 200 mM KCl, 4 mM 1,4-dithioerythritol, and $Mg(OAc)_2$, as indicated. Final buffer concentration was fourfold lower in the incubation mixtures.

Results

Characterization of the Components of the System. The binding of C-G-[^{3}H]A-[^{3}H]A to $tRNA^{Phe}$ was assayed by the equilibrium dialysis process as outlined before (Schwarz et al., 1974). Complete equilibrium was achieved within 2.5 h with a new type of membrane (Table I). The permeability was not affected at the protein concentrations used under assay conditions. Nuclease activity was completely removed from $EF\text{-}T_u$ by three recrystallizations of the binary complex $EF\text{-}T_u$·GDP. 30S ribosomes prepared according to the method of Noll et al. (1973) showed no nuclease activity over a period of 24 h at 0–4

TABLE I: Time Dependence of C-G-A-A Equilibration Through the Dialysis Membrane.[a]

Time (min)	C-G-A-A (pmol)	
	Compartment I	Compartment II
0	0	223 ± 1
30	41 ± 1	182 ± 1
60	94 ± 1	129 ± 1
120	110 ± 1	113 ± 1
180	110 ± 1	110 ± 1

[a] In a total volume of 100 μl both compartments contained 25 μl of buffer. Compartment II contained in addition 230 pmol of C-G-[^{3}H]A-[^{3}H]A. Within the incubation times used, the membrane was not permeable to $(U)_8$ as tested with [^{3}H]$(U)_8$.

TABLE II: Nonspecific Binding of C-G-A-A to the Assay System Containing Poly(U).[a]

Components	C-G-A-A Bound (pmol)
100 pmol of 30S	1.9
5 μg of poly(U)	24.0
5 μg of poly(U) + 100 pmol of 30S	385.0
10 μg of $(U)_8$ + 100 pmol of 30S	1.0

[a] The following incubation mixture (100 μl) was incubated at 0 °C for 30 min: 25 μl of buffer, 1 nmol of C-G-[^{3}H]A-[^{3}H]A, and the components listed. The final Mg^{2+} concentration was 10 mM. Complex formation was assayed by the nitrocellulose filter technique.

TABLE III: Specificity of C-G-A-A Binding.[a]

Components	Residual C-G-A-A Bound to the Complete System (pmol)
50 pmol of 30S	9.6
50 pmol of 30S + 25 nmol of $(U)_8$	9.7
50 pmol of 30S + 200 pmol of Phe-tRNA	9.1
50 pmol of 30S + 400 pmol of EF-T_u·GTP	10.8
50 pmol of 30S + 200 pmol of Phe-tRNA + 400 pmol of EF-T_u·GTP	9.6
25 nmol of $(U)_8$ + 200 pmol of Phe-tRNA + 400 pmol of EF-T_s·GTP	6.4
50 pmol of 30S + 25 nmol $(U)_8$ + 200 pmol of Phe-tRNA	6.2
50 pmol of 30S + 25 nmol of $(U)_8$ + 200 pmol of Phe-tRNA + 400 pmol of EF-T_u·GTP	0.1

[a] Compartment I contained, in a total volume of 100 μl, 25 μl of buffer, 100 nmol of GTP, and the components listed. Compartment II contained the complete system and 250 pmol of C-G-[^{3}H]A-[^{3}H]A. The Mg^{2+} concentration was 10 mM. After 3 h at 4 °C the radioactivity of each compartment was determined as described under Methods.

°C when tested with [^{3}H]poly(U) as substrate. Initially $(U)_{50-100}$ was used to stimulate the binding of Phe-tRNA to 30S ribosomes. As revealed in the nitrocellulose-filter assay, the 30S·$(U)_{50-100}$ complex showed a high degree of nonspecific

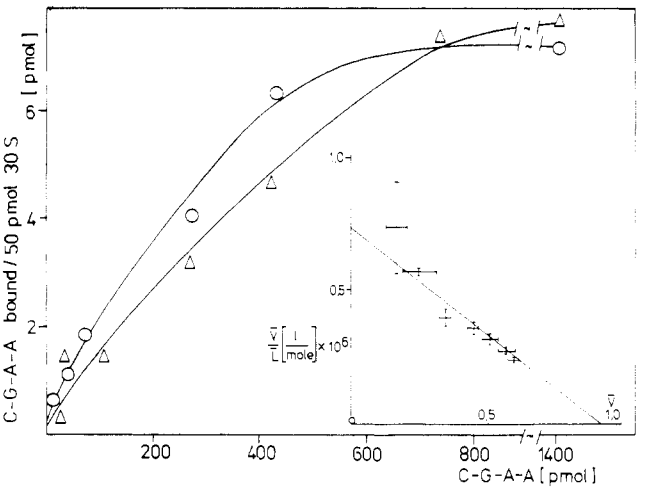

FIGURE 2: Binding of C-G-A-A to Phe-tRNA. Rearrangement of the Phe-tRNA structure is monitored by C-G-A-A binding, which is calculated from equilibrium dialysis experiments. In the absence of EF-T_u·GTP (-Δ—Δ-) both chambers contained (in a total volume of 100 μl) 25 μl of buffer, 50 pmol of 30S subunits, 200 pmol of Phe-tRNA, and a final Mg^{2+} concentration of 10 mM. One chamber contained in addition 25 nmol of $(U)_8$ and 250 pmol of labeled C-G-[^{3}H]A-[^{3}H]A. For the "enzymatic" binding (-O—O-) both chambers were supplemented with 400 pmol of EF-T_u·GDP and 100 pmol of GTP. The insert gives the Scatchard plot for the enzymatic binding. The straight line is the theoretical curve calculated by linear regression from assorted data points with $f_i = \bar{V}(\bar{V}/L)$.

C-G-A-A binding. When we substituted $(U)_{50-100}$ by $(U)_8$ this nonspecific binding was abolished (Table II).

Specificity of C-G-A-A Binding. The controls listed in Table III were performed in order to show that the binding of C-G-A-A occured specifically to the T-ψ-C-G sequence of the Phe-tRNA and not to any other component of the system. To minimize artifacts, compartment II always contained the complete system, whereas compartment I was supplied with the components indicated in the table. Only small amounts of the oligonucleotide C-G-A-A were bound to 30S, $(U)_8$, Phe-tRNA and to the ternary complex EF-T_u·GTP·Phe-tRNA. In the presence of $(U)_8$ the difference between the complete system and the 30S·$(U)_8$·Phe-tRNA complex was only 6.4 pmol, i.e., 4.4 pmol was bound to this complex; 4.6 pmol was bound to the ternary complex EF-T_u·GTP·Phe-tRNA in the presence of $(U)_8$ (Table III).

Using saturating amounts of C-G-[^{3}H]A-[^{3}H]A and optimal Mg^{2+} concentrations (10 mM), 6–7 pmol of C-G-A-A was bound to the $tRNA^{Phe}$ in a reaction mixture containing 50 pmol of 30S ribosomes (Figure 2). This corresponds well to the amount of Phe-tRNA bound "enzymatically" or "nonenzymatically" to the 30S·$(U)_8$ complex as assayed by the nitrocellulose filter technique (Table IV).

The data for C-G-[^{3}H]A-[^{3}H]A enzymatic binding were analyzed according to Scatchard (1949) and yielded a straight line (insert to Figure 2). The association constant and number of binding sites were calculated by linear regression from the slope and the intercept: $K_{ass} = 7.3 \times 10^5\ M^{-1}$, $n = 0.93$, correlation coefficient $r = 0.95$. These data prove the existence of only one binding site with a high association constant, which is strong support for the assumed T-ψ-C-G·C-G-A-A complex.

Inhibition of Complex Formation By the 50S Ribosomal Subunits. The inhibition of complex formation by 50S ribosomal subunits is depicted in Figure 3. The addition of equimolar amounts of 50S subunits reduced the complex formation to 12% of the initial value. This strong inhibition by the larger ribosomal subunit can be understood if one realizes that the

TABLE IV: $(U)_8$ Stimulated Binding of EF-T_u·GTP·[^{3}H]Phe-tRNA and [^{3}H]Phe-tRNA to 30S Ribosomes (50 pmol) at 10 mM Mg^{2+}.[a]

Time (min)	Phe-tRNA bound (pmol)	
	EF-T_u absent	EF-T_u present
0	0.2	0.5
60	5.4	6.1
120	5.8	6.9
180	6.1	8.4

[a] The incubation mixtures for the "nonenzymatic" binding contained, in a total volume of 100 μl, 25 μl of buffer, 50 pmol of 30S subunits, 200 pmol of [^{3}H]Phe-tRNA (charged to 25%, sp act. 1000 Ci/mol), and 25 nmol of $(U)_8$. For the "enzymatic" binding, 400 pmol of EF-T_u·GDP and 100 nmol of GTP were added. The samples were incubated at 4 °C for 30 min. The [^{3}H]Phe-tRNA bound was determined by the nitrocellulose filter assay.

TABLE V: Binding of C-G-A-A to $tRNA^{Phe}$ in the Absence of 30S Ribosomes.[a]

Components	pmol C-G-A-A Bound at	
	0.25 mM $(U)_8$	0.50 mM $(U)_8$
$(U)_8$	1.7	2.0
Phe-tRNA + $(U)_8$	2.1	8.6
$(U)_8$ + Phe-tRNA + EF-T_u·GTP	8.3	9.0

[a] Compartment I contained in a total volume of 100 μl of buffer, Phe-tRNA, or EF-T_u·GTP·Phe-tRNA, while compartment II contained in addition $(U)_8$ as indicated and 250 pmol of C-G-|^{3}H|A-|^{3}H|A.

C-G-A-A sequence within the 5S rRNA of the 50S ribosome is optimally oriented towards complex formation to T-ψ-C-G (Erdmann et al., 1973).

Requirement of EF-T_u and Mg^{2+}. The Mg^{2+} dependence of the tetranucleotide binding to the complex 30S·$(U)_8$·EF-T_u·GTP·Phe-tRNA (Figure 4) gives a hyperbolic type binding curve. If EF-T_u is omitted, a sigmoidal binding of C-G-A-A as a function of Mg^{2+} concentration is observed with a point of inflection at 6 mM Mg^{2+}. It, therefore, can be concluded that EF-T_u·GTP, as well as higher Mg^{2+} concentrations, support the rearrangement of the $tRNA^{Phe}$, whereas GTP may be replaced by GMP-P(NH)P within the ternary complex (data not shown). This proves that, at low Mg^{2+} concentrations, the rearrangement of the $tRNA^{Phe}$ is not dependent on the GTP-catalyzed removal of the elongation factor EF-T_u (Yokosawa et al., 1973). At Mg^{2+} concentrations higher than 8 mM the same amount of C-G-A-A was bound to $tRNA^{Phe}$ compared to the complex EF-T_u·GTP·Phe-tRNA (Figure 4). This may be regarded as further evidence that at high Mg^{2+} concentrations the change in the $tRNA^{Phe}$ structure is not dependent on EF-T_u.

Dependence of Rearrangement of $tRNA^{Phe}$ Structure on Codon–Anticodon Interaction. The data presented above do not discriminate between the possibilities that the rearrangement of the $tRNA^{Phe}$ is due to the coded binding of the $tRNA^{Phe}$ to the ribosomes or to codon–anticodon interaction. Therefore, the binding of C-G-A-A to $tRNA^{Phe}$ was examined since it had been shown by several authors (Högenauer, 1970; Uhlenbeck et al., 1970; Gassen et al., 1972) that a complex is

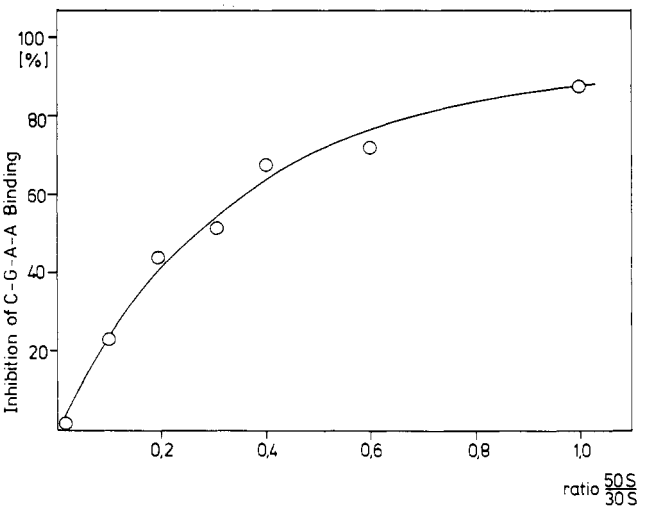

FIGURE 3: Inhibition of C-G-A-A binding by the 50S ribosomal subunit. Compartment I contained (in a total volume of 100 μl) 25 μl of buffer, 50 pmol of 30S subunits, 400 pmol of EF-T_u·GDP, 200 pmol of Phe-tRNA, and 50 S subunits as indicated. Compartment II contained an additional 25 nmol of $(U)_8$ and 250 pmol of C-G-[^{3}H]A-[^{3}H]A. Other experimental conditions were the same as indicated under Figure 2.

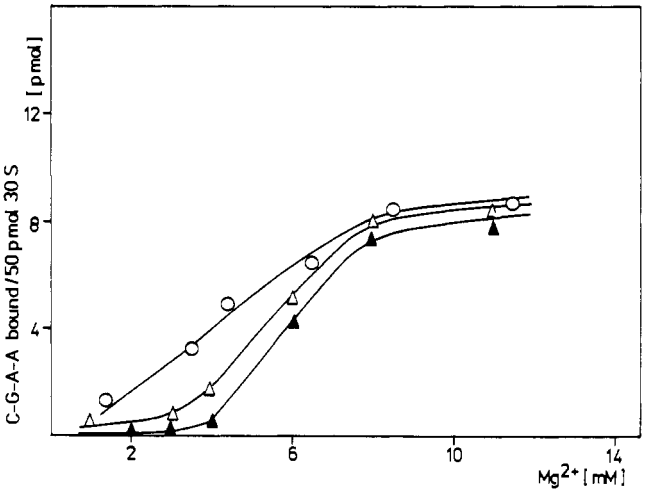

FIGURE 4: Mg^{2+} dependence of the binding of C-G-A-A to Phe-tRNA bound to 30S ribosomes in the absence and presence of EF-T_u·GTP. Compartment I contained (in a total volume of 100 μl) 25 μl of buffer, 50 pmol of 30S subunits, 200 pmol of Phe-tRNA (-Δ—Δ-) or $tRNA^{Phe}$ (-▲—▲-) or 400 pmol of EF-T_u·GDP, 100 nmol of GTP, and 200 pmol of Phe-tRNA (-O—O-). Compartment II was supplemented with 25 nmol of $(U)_8$ and 250 pmol of C-G-[^{3}H]A-[^{3}H]A.

formed between a tRNA and a trinucleoside diphosphate in the absence of ribosomes with K_{ass} of 10^3–10^4 M^{-1}. The rather low association constant naturally implies that higher concentrations of $(U)_8$ should be used in the absence of ribosomes than when they are present. At $(U)_8$ concentrations which were saturating in the presence of 30S subunits, no rearrangement of the $tRNA^{Phe}$ could be detected in the absence of 30S ribosomes. However, at higher concentrations of $(U)_8$ (0.5 mM) almost the same amount of tetranucleotide was bound to the $tRNA^{Phe}$ as compared to the ribosomal system (Table V). With the ternary complex EF-T_u·GTP·Phe-tRNA 0.25 mM $(U)_8$ was sufficient to get a comparable level of oligonucleotide binding.

To obtain further support for the proposal that the rearrangement of the $tRNA^{Phe}$ is caused by codon–anticodon complex formation the Mg^{2+} dependence in the presence of

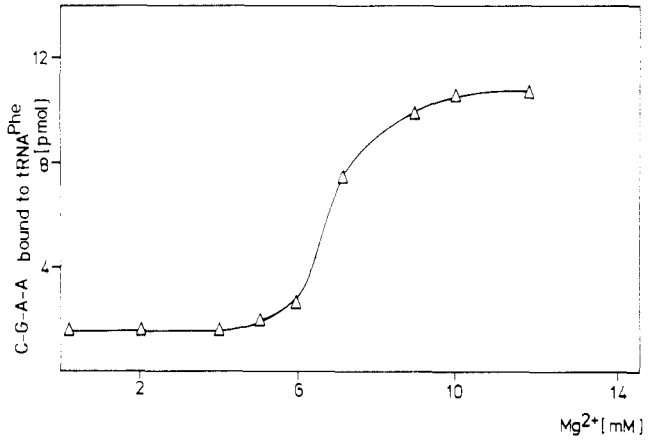

FIGURE 5: Mg^{2+} dependence of the binding of C-G-A-A to $tRNA^{Phe}$ in the absence of 30S ribosomes. Compartment I contained (in a total volume of 100 μl) 25 μl of buffer and 200 pmol of $tRNA^{Phe}$. In addition compartment II contained 50 nmol of $(U)_8$ and 250 pmol of C-G-[^{3}H]A-[^{3}H]A.

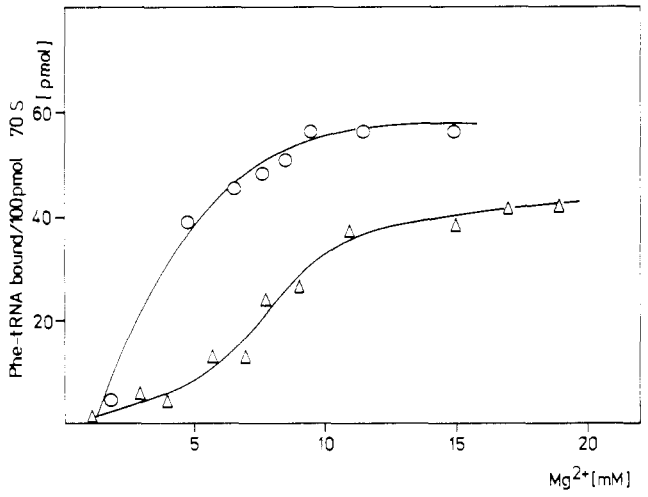

FIGURE 6: Mg^{2+} dependence of the "nonenzymatic" and "enzymatic" poly(U)-coded binding of [^{3}H]Phe-tRNA to 70S ribosomes. The incubation mixtures contained (in a total volume of 100 μl) 25 μl of buffer, 25 pmol of 70S ribosomes, 200 pmol of [^{3}H]Phe-tRNA, 5 μg of poly(U), 100 nmol of GTP, and 400 pmol of EF-T_u·GDP (-O—O-). For the "nonenzymatic" binding assays, GTP and EF-T_u·GDP were omitted (-Δ—Δ-). The samples were incubated for 40 min at 0 °C. The amount of [^{3}H]Phe-tRNA bound was measured by the nitrocellulose filter assay. Blanks (without ribosomes) were subtracted.

excess $(U)_8$ was tested (Figure 5). The binding of C-G-A-A to the $tRNA^{Phe}$·$(U)_8$ complex again shows a strong cooperative Mg^{2+} effect with half-saturation between 5 and 7 mM Mg^{2+}. One may, therefore, conclude that codon–anticodon recognition causes a change in the $tRNA^{Phe}$ structure such that the correct $tRNA^{Phe}$ becomes bound to the C-G-A-A sequence via the now exposed T-ψ-C-G sequence.

Relation of the Rearrangement to the Coded Binding of Phe-tRNA to 30S and 70S Ribosomes. The rearrangement of the $tRNA^{Phe}$ exposing the T-ψ-C-G sequence of binding to the 5S rRNA of the 50S subunit should be reflected in a difference in binding to either the 70S or the 30S subunit (Figure 6). Therefore, Phe-tRNA binding to 30S and 70S ribosomes was investigated as a function of Mg^{2+} concentration in the presence and absence of EF-T_u. A hyperbolic type curve is obtained for the "enzymatic" binding, whereas in the "nonenzymatic" binding a sigmoidal curve results with half saturation at 6 mM Mg^{2+}. This indicates that the structure

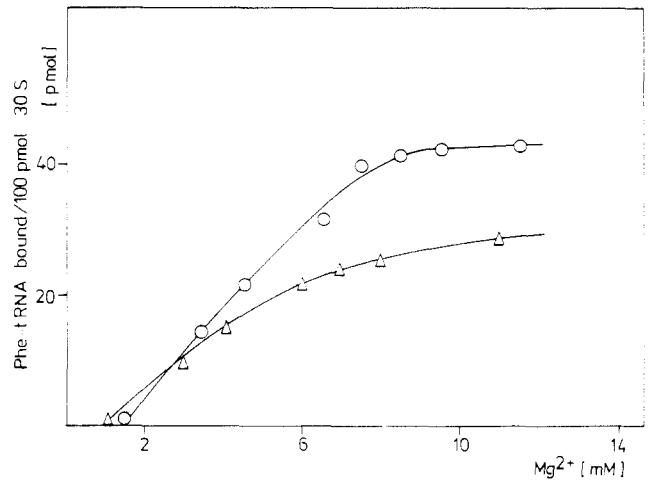

FIGURE 7: Mg^{2+} dependence of the "nonenzymatic" and "enzymatic" binding of [^{3}H]Phe-tRNA to 30S ribosomes. Reaction conditions were as described in the legend to Figure 6, except that the 70S ribosomes were replaced by the 30S ribosomes [(-Δ—Δ-) "nonenzymatic" binding, (O—O-) "enzymatic" binding].

of the $tRNA^{Phe}$ is changed, displaying the T-ψ-C-G sequence for binding to the 50S subunit. Thus, the Mg^{2+} dependence of the "nonenzymatic" binding of Phe-tRNA to 70S ribosomes resides in a structural change of the $tRNA^{Phe}$. The lower plateau value reached in the "nonenzymatic" binding is caused by competition of uncharged $tRNA^{Phe}$ (Levin, 1970).

With 30S subunits in both cases ("enzymatic" and "nonenzymatic") only hyperbolic curves are found, because exposure of the T-ψ-C-G sequence does not create an additional binding site (Figure 7).

Thus, the C-G-[^{3}H]A-[^{3}H]A binding experiments as measured by equilibrium dialysis are further supported by a comparison of "enzymatic" and "nonenzymatic" Phe-tRNA binding to 70S and 30S ribosomes.

Discussion

The first experiments to measure the codon-dependent binding of C-G-A-A to $tRNA^{Phe}$ were hampered by the fact that the membranes used for the dialysis were not readily permeable to the tetranucleoside triphosphate. Equilibration times of 24–36 h, together with traces of nuclease contamination of the elongation factor and 30S ribosomes, caused considerable degradation of the oligonucleotides in the test system. These difficulties were overcome by use of a new membrane and by intensive purification of EF-T_u and 30S ribosomes. The second problem we faced was the nonspecific binding of C-G-A-A to the $(U)_{50-100}$·30S complex, as measured by the nitrocellulose filter technique (Table II). Replacement of $(U)_{50-100}$ by $(U)_8$ abolished this nonspecific binding, while stimulation of phenylalanyl-tRNA binding was maintained at 8 pmol/50 pmol of 30S ribosomes.

For the experiments, $tRNA^{Phe}$ was used which was charged to 25% with phenylalanine. This low degree of charging should not influence the T-ψ-C-G·C-G-A-A interaction, since by NMR measurements it had been shown that there is no structural difference between Phe-tRNA and $tRNA^{Phe}$ (Wong et al., 1973).

The crucial point upon which the interpretation of our data rests is the assumption that C-G-A-A binds specifically to the T-ψ-C-G sequence of $tRNA^{Phe}$. Although final proof cannot be given, acceptance of this assumption appears to be inevitable. In the dialysis experiments, the contents of the two

chambers differed by only one component, which served to reduce blank values (Table III). The amount of Phe-tRNA bound to the 30S·$(U)_8$ complex and the amount of C-G-A-A bound to 30S·$(U)_8$·Phe-tRNA were almost identical. Analysis of C-G-A-A binding to the 30S·$(U)_8$·Phe-tRNA complex yields only one binding site/complex with an association constant of 7.3×10^5 M^{-1}. The magnitude of the association constant is in the expected range for a complex between two tetranucleoside triphosphates, when compared with the constants determined for antiparallel complementary anticodons (Högenauer, 1974). Finally, inhibition of oligonucleotide binding by 50S particles also supports specific complex formation. From the above reasoning, we are convinced that the C-G-A-A binds to the T-ψ-C-G loop of $tRNA^{Phe}$ and that this binding represents a parameter that can be used to follow structural changes within the $tRNA^{Phe}$.

It now can be decided whether this rearrangement is a consequence of Phe-tRNA–30S ribosome interaction or is solely due to codon–anticodon complex formation. In the ribosome-free system, no exposure of T-ψ-C-G can be detected at low concentration of $(U)_8$ (0.25 mM), while at higher concentrations (0.5 mM) exposure occurs to a considerable extent (Table V). This dependence on high $(U)_8$ concentration corresponds well to the independently determined association constant for $(U)_3$ (Uhlenbeck et al., 1970; Gassen et al., 1972). Although quantitative interpretations are not possible at this stage, it is obvious that conformational alterations that result in exposure of T-ψ-C-G are controlled solely by codon–anticodon interaction. In the presence of EF-T_u, a change of $tRNA^{Phe}$ conformation occurs at low concentrations of $(U)_8$ and is indistinguishable from that shown by the complete system which includes 30S ribosomes. This can only be understood if a high association constant of codon binding to the EF-T_u·GTP·Phe-tRNA complex is assumed. Furthermore, codon-induced change of $tRNA^{Phe}$ conformation is unrestricted by EF-T_u in the ternary complex.

The Mg^{2+} dependence of $tRNA^{Phe}$ rearrangement is cooperative both in the presence and absence of 30S ribosomes, while EF-T_u abolishes cooperativity. This dependence cannot be due to binding of Mg^{2+} to 30S ribosomes, because these are saturated at 2 mM Mg^{2+} (Weiss and Morris, 1973; Kimes and Morris, 1973) and 30S ribosome conformation does not depend on Mg^{2+} after reactivation (Ginzburg and Zamir, 1975). However, the tertiary structure of tRNA is influenced by Mg^{2+} (Cohn et al., 1968; Schreier and Schimmel, 1974; Römer and Hach, 1975). The interaction of the T-ψ-C-G loop and the dihydrouridine loop is stabilized by Mg^{2+} ions which are bound with high affinity (Lynch and Schreier, 1974). Whether this binding occurs in a cooperative manner is as yet undecided (Römer and Hach, 1975). Even though this binding appears to be a likely one to control T-ψ-C-G exposure, the measured association constant is much too high (Römer and Hach, 1975). For the same reason weaker binding sites which are associated with helix coil transitions of stem regions (Schreier and Schimmel, 1974) also cannot account for the observed Mg^{2+} dependence. Therefore, none of the presently known Mg^{2+} binding sites covers the concentration range in question and can be associated with the control of codon induced $tRNA^{Phe}$ rearrangement.

From the data presented, it is evident that the coded binding of an AA-tRNA must be divided into at least two steps. The initial event is the recognition of the appropriate AA-tRNA by the mRNA codon bound to the 30S ribosomal decoding site. As a result of codon–anticodon complex formation, the tertiary structure of the tRNA is altered and the tRNA is bound to the 50S subunit. This sequence of events appears to be necessary, otherwise all tRNAs would compete for the same binding site on the 50S subunit. Furthermore, the binding of the tRNA to the 30S, as well as to the 50S subunit, may help to stabilize the 70S ribosome during the elongation cycle. Naturally, additional changes in the tertiary structure of either aminoacyl- or peptidyl-tRNA may occur during defined steps in protein synthesis, as has been postulated by Kurland et al. (1975).

RNA double-helix formation taking place during codon–anticodon complex formation has to be the dominating event for the rearrangement of the tRNA structure. The implications of this complex formation on the structure of the anticodon loop has been suggested (Fuller and Hodgson, 1967; Woese, 1970). The allosteric effect of codon–anticodon interaction, i.e., the transduction of the structural alterations through the anticodon stem to the T-ψ-C-G loop and the dihydrouridine loop, is more difficult to understand. The first temperature-dependent change in the structure of $tRNA^{Val1}$ (Privalov et al., 1975) and $tRNA^{fMet}$ (Crothers et al., 1974) is due to a change in the interaction of the T-ψ-C-G loop and dihydrouridine loop. In the three-dimensional structure of $tRNA^{Phe}$ from yeast as determined by x-ray crystallography, the stabilization of the two loops is brought about by rather weak "non-Watson–Crick" type hydrogen bonds (Kim et al., 1973; Robertus et al., 1974). Therefore, minimal changes in the structure of the anticodon stem could influence the geometry of the T-ψ-C-G loop. Structural changes in a macromolecule far from the point of substrate attachment are well known for proteins and have been described in molecular terms for hemoglobin (Perutz, 1970). With nucleic acids, one is tempted to think of the intercalation of ethidium bromide into DNA, which causes unwinding of the twisted circular structure even when the ratio of dye molecules/base pair is quite low (Bauer and Vinograd, 1968). In summary, we believe that the evidence presented strongly supports a codon-dependent change of the $tRNA^{Phe}$ structure and may represent in more general terms a first example for allosteric rearrangements in nucleic acids.

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