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tRNA Binding Sites of Ribosomes from *Escherichia coli*†

Roland Lill, James M. Robertson, and Wolfgang Wintermeyer*

ABSTRACT: 70S tight-couple ribosomes from *Escherichia coli* were studied with respect to activity and number of tRNA binding sites. The nitrocellulose filtration and puromycin assays were used both in a direct manner and in the form of a competition binding assay, the latter allowing an unambiguous determination of the fraction of ribosomes being active in tRNA binding. It was found that, in the presence of poly(U), the active ribosomes bound two molecules of *N*-AcPhe-tRNA^{Phe}, one in the P and the other in the A site, at Mg^{2+} concentrations between 6 and 20 mM. A third binding site in addition to P and A sites was observed for deacylated tRNA^{Phe}. At Mg^{2+} concentrations of 10 mM and below, the occupancy of the additional site was very low. Dissociation of tRNA from this site was found to be rather fast, as compared to both P and A sites. These results suggest that the additional site during translocation functions as an exit site, to which deacylated tRNA is transiently bound before leaving the ribosome. Since tRNA binding to this site did not require the presence of poly(U), a function of exit site bound tRNA in the fixation of the mRNA appears unlikely. Both the affinity and stability of binding to the additional site were found lower for the heterologous tRNA^{Phe} from yeast as compared to the homologous one. This difference possibly indicates some specificity of the *E. coli* ribosome for tRNAs from the same organism.

The generally accepted model of the ribosomal elongation cycle is based upon the existence of two tRNA binding sites on the ribosome: one for peptidyl-tRNA (P site) and another for aminoacyl-tRNA (A site). From the P site bound peptidyl-tRNA, the peptide is transferred to the aminoacyl-tRNA to yield peptidyl-tRNA in the A site and deacylated tRNA in the P site. During the subsequent translocation step, the A site bound peptidyl-tRNA is translocated to the P site, and the deacylated tRNA is released.

Additional tRNA binding sites other than the two canonical ones have been discussed repeatedly. For instance, an entry (Hardesty et al., 1969) or recognition (Lake, 1977) site has been proposed, from which the aminoacyl-tRNA reaches the A site only after GTP hydrolysis and codon recognition have taken place. Since at least the anticodon region of the tRNA in both recognition and A sites is bound to the same site on the ribosome, such a recognition site does not constitute an independent, nonoverlapping site. A third site functioning as an exit site, from which deacylated tRNA leaves the ribosome after translocation, has been reported for eucaryotic ribosomes (Wettstein & Noll, 1965).

For *Escherichia coli* ribosomes, the existence of a third, independent site being accessible only for deacylated tRNA has been reported recently by Rheinberger et al. (1981). Furthermore, these authors reported that binding of the peptidyl-tRNA analogue *N*-AcPhe-tRNA^{Phe} to the P site excluded binding of *N*-AcPhe-tRNA^{Phe} to the A site and vice versa ("exclusion principle"). While the existence of the third binding site for deacylated tRNA was confirmed by some authors (Grajevskaja et al., 1982; Kirillov et al., 1983), it was doubted by others, because the additional binding could not be detected in equilibrium centrifugation experiments (Schmitt et al., 1982) and because stoichiometric release of deacylated tRNA was observed within one round of translocation in a column-bound poly(U)-ribosome system (Spirin, 1984). Concerning the binding of peptidyl-tRNA, Kirillov & Semenkov (1982) presented evidence for the simultaneous binding of two molecules of *N*-AcPhe-tRNA^{Phe} to one ribosome.

Since the exact knowledge of the number of tRNA binding sites is fundamental for any study of ribosome function, we reexamined the controversial issue and studied the tRNA binding properties of tight-couple ribosomes from *E. coli* in some detail. For comparisons, some experiments were also performed with ribosomes prepared according to Rheinberger & Nierhaus (1980). Binding experiments were carried out with deacylated tRNA^{Phe} and *N*-AcPhe-tRNA^{Phe} from both *E. coli* and yeast. To escape a possible misinterpretation of saturation titration plateaus, a competition binding assay was developed which allowed an independent determination of the

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tRNA binding activity of the ribosomes.

Materials and Methods

Ribosomes, tRNA, and Elongation Factor Tu. The isolation of tight-couple 70S ribosomes from *E. coli* MRE 600 cells and of $tRNA^{Phe}$ (charging capacity 1.5–1.7 nmol of Phe/A_{260} unit) from brewers' yeast tRNA (Boehringer Mannheim), as well as aminoacylation and acetylation of the yeast $tRNA^{Phe}$, was described previously (Robertson & Wintermeyer, 1981). $tRNA^{Phe}$ from *E. coli* (charging capacity 1.6 nmol of Phe/A_{260} unit; Boehringer Mannheim) was aminoacylated and acetylated according to a published method (Rappoport & Lapidot, 1974). [^{14}C]Phe-$tRNA^{Phe}$ and *N*-Ac[^{14}C]Phe-$tRNA^{Phe}$ (504 Ci/mol, if not stated otherwise) were charged to 70–80% and 80–95%, respectively, calculated on the basis of 1.75 nmol/A_{260} unit. The latter figure was also used to calculate tRNA concentrations. Ribosome concentrations were calculated by assuming 23 pmol/A_{260} unit. Elongation factor Tu (EF-Tu) from *E. coli* was a gift of A. Parmeggiani (Chinali et al., 1977).

Preparation of ^{32}P-Labeled $tRNA^{Phe}$. Labeling was performed by exchanging the 5′-phosphate of $tRNA^{Phe}$ from either yeast or *E. coli* with [^{32}P]phosphate. Five A_{260} units of $tRNA^{Phe}$ in 0.1 mL of 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl), pH 8.0, were incubated with 3–5 units of alkaline phosphatase from calf intestine (Boehringer Mannheim) for 45 min at 50 °C. After extraction of the protein with phenol followed by ether extraction, the dephosphorylated tRNA was collected by ethanol precipitation.

Phosphorylation was performed with polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP in 0.2 mL of 25 mM Tris·HCl, pH 8.0, containing 10 mM $MgCl_2$, 2 mM spermine, 5 mM dithiothreitol, and bovine serum albumin (10 μg/mL) at 37 °C. Small tRNA fragments are preferentially labeled in the kinase reaction, leading to a tRNA preparation heavily contaminated with radioactively labeled oligonucleotides, also if only minute amounts are present. This problem was avoided by conducting the kinase reaction in two steps: It was started by a 20-min incubation with 20 units of the enzyme and 3 nmol of unlabeled ATP (ATP/tRNA = 0.3); then 18 nmol of [γ-^{32}P]ATP (about 0.1 mCi) and 60 units of the enzyme were added, and the incubation was continued for another 90 min, after which time the plateau of ^{32}P incorporation was usually reached, as determined by trichloroacetic acid precipitation. The labeled tRNA was isolated by ethanol precipitation and gel filtration on Sephadex G-25.

The purity of ^{32}P-labeled tRNA was checked by polyacrylamide gel electrophoresis in the presence of urea and autoradiography. If more than 5% of the radioactivity was found in material smaller than tRNA, the tRNA was purified to homogeneity by preparative gel electrophoresis. The charging capacity of the labeled tRNA was found to be above 1.5 nmol of Phe/A_{260} unit. Finally, in order to avoid any significant contamination with unlabeled tRNA molecules lacking a 5′-phosphate, the labeled $tRNA^{Phe}$ was diluted 10-fold with untreated $tRNA^{Phe}$ to a specific radioactivity of about 500 cpm/pmol.

Formation and Analysis of tRNA–Ribosome Complexes. For the binding experiments, the following buffers were used: (A) 50 mM Tris·HCl, pH 7.5, 50 mM KCl, 90 mM NH_4Cl, 1 mM dithioerythritol, and 20 mM magnesium acetate; (B) 50 mM Tris·HCl, pH 7.5, 30 mM KCl, 30 mM NH_4Cl, 1 mM dithioerythritol, and 6, 10, or 15 mM magnesium acetate, as indicated. Poly(U) was present at a concentration of 1 A_{260} unit/mL if not stated otherwise.

Titrations were performed by varying the tRNA concentration as indicated, keeping the ribosome concentration constant (0.2–0.5 μM). The reaction mixtures (approximately 50 μL) were incubated at 20 °C for 30, 60, 90, or 120 min at 20, 15, 10, or 6 mM Mg^{2+}, respectively, in order to take into account the strong Mg^{2+} dependence of the kinetics of A site binding. Subsequently, the indicator reactions were started by the addition (5 μL) of 15% (relative to ribosomes) of either *N*-AcPhe-$tRNA^{Phe}$ (P site) or ternary complex (A site) and analyzed by the puromycin or filtration assay (see below) after incubation at 20 °C for 15 min or 30 s, respectively. The ternary complex was prepared by incubating Phe-$tRNA^{Phe}$ with a stoichiometric amount of EF-Tu in the presence of 0.3 mM GTP, 1 mM ATP, 6 mM phosphoenolpyruvate, and pyruvate kinase (10 μg/mL) for 10 min at 20 °C in the respective titration buffer.

tRNA–ribosome complexes were isolated by directly applying the reaction mixtures to nitrocellulose filters (Sartorius SM 11306), without prior dilution, if not stated otherwise. The standard puromycin assay was performed by the addition of 5 μL of 10 mM puromycin followed by an incubation for 20 min at 37 °C; for the indicator reaction, the incubation with puromycin was stopped after 7 min. The extraction procedure of *N*-AcPhe-puromycin and also the determination of charged tRNA by precipitation with trichloroacetic acid were described previously (Robertson & Wintermeyer, 1981).

Evaluation of the Indicator Titrations. From the indicator titrations, the concentration of ribosomes being active in tRNA binding was determined by computer fitting. The models used were based upon the fact that there is no significant binding to other sites before the P site is occupied. Thus, P site binding is evaluated with the assumption that the ribosomal tRNA binding sites are filled in a sequential manner. Similarly, binding to the A site, since it becomes significant only after the P site has been filled, can be treated as a single-site problem.

Indicator Titration of the P Site. The titration of poly(U)-programmed, vacant ribosomes (R) with tRNA (T) (denoting either deacylated $tRNA^{Phe}$ or *N*-AcPhe-$tRNA^{Phe}$ of relatively low specific radioactivity; see Results) and the determination of unoccupied P sites by subsequent binding of the indicator *N*-AcPhe-$tRNA^{Phe}$ (I) are depicted in the scheme shown in eq 1a (K_P and K_{IP} are equilibrium constants of P site binding of tRNA and indicator tRNA, respectively):

$$\begin{array}{ccc} R + T & \underset{K_P}{\rightleftharpoons} & RT_P \\ + & & \\ I & & \\ {\scriptstyle K_{IP}}\downarrow & & \\ RI_P & & \end{array} \qquad (1a)$$

Provided the second incubation is short relative to the exchange rate of the prebound tRNA, binding of the indicator tRNA will be restricted to P sites remaining unoccupied in the first incubation. Since the total concentration of ribosomes, c_{R^0}, is kept constant (see above), the fractional saturation of the P sites, n_P, may be defined as the concentration of ribosomes occupied in the P site relative to c_{R^0}. With that, n_P for a given tRNA concentration (c_{T^0}) is obtained from eq 2a, which is derived from the law of mass action:

$$n_P(c_{T^0}) = [L - (L^2 - 4c_{T^0}c_{R^0})^{1/2}]/(2c_{R^0}) \qquad (2a)$$

with

$$L = c_{T^0} + c_{R^0} + 1/K_P$$

where c_{T^0} and c_{R^0} are the total concentrations of T and R, respectively. Then, the concentration of unoccupied P sites,

c_{UP}, available for binding the indicator tRNA is given by

$$c_{UP}(c_{T^0}) = [1 - n_P(c_{T^0})]c_{R^0} \quad (3a)$$

During the subsequent incubation with indicator tRNA, the total concentration of indicator tRNA, c_{I^0}, is constant, and the concentration of unoccupied P sites, c_{UP}, varies. Therefore, the fractional saturation of the P sites with indicator tRNA, n_{IP}, is defined as the concentration of bound indicator tRNA relative to c_{I^0}. Similar to eq 2a, n_{IP} can be calculated from

$$n_{IP}(c_{T^0}) = \{M - [M^2 - 4c_{UP}(c_{T^0})c_{I^0}]^{1/2}\}/(2c_{I^0}) \quad (2b)$$

with

$$M = c_{I^0} + c_{UP}(c_{T^0}) + 1/K_{IP}$$

n_{IP} is related to the amount of puromycin-reactive indicator tRNA, I_P, according to

$$I_P(c_{T^0}) = I_{max}n_{IP}(c_{T^0}) + I_B \quad (4a)$$

I_{max} denotes the signal observed when all indicator tRNA is bound to the P site, offset by a blank value, I_B, for no binding of indicator.

Equation 4a served to estimate the concentration of ribosomes being active in binding tRNA to the P site by a Gauss–Newton iteration procedure (Graybill, 1969). Optimal fits were obtained when the parameters I_{max} and I_B were also fitted. The binding constants K_P and K_{IP} were determined in equilibrium titrations by using the fluorescence as well as the nitrocellulose filtration technique; details will be described elsewhere. Here, for buffer A, the binding constants of 1.6×10^8 and 5.5×10^8 M^{-1} were used for deacylated $tRNA^{Phe}$ and N-AcPhe-$tRNA^{Phe}$, respectively; for buffer B at 10 mM Mg^{2+}, the respective data are 1.0×10^8 and 5.0×10^8 M^{-1}.

The estimated parameters varied by less than 5% due to the error in the binding constants (20%). Also, the scattering of the binding data (less than 8%) did not influence the results significantly.

Indicator Titration of the A Site. The evaluation is derived only for the case where the A site is the second site to be filled. Titrations in which the A site is occupied subsequently to or concomitantly with the E site (see Results) can be treated in an analogous, although somewhat more involved manner.

According to the sequential binding model, the calculation of the actual concentration of tRNA available for binding to the A site has to account for P site binding, which precedes A site binding:

$$\begin{array}{ccccc} R + T & \underset{K_P}{\rightleftharpoons} & RT_P + T & \underset{K_A}{\rightleftharpoons} & RT_PT_A \\ & & + & & \\ & & I & & \\ & & \Big\| K_{IA} & & \\ & & RT_PI_A & & \end{array} \quad (1b)$$

The fractional saturation of the P site again can be calculated from eq 2a. The concentration of tRNA remaining available for A site binding, c_{TA}, then is given by the difference of total and P site bound tRNA:

$$c_{TA}(c_{T^0}) = c_{T^0} - n_P(c_{T^0})c_{R^0} \quad (5)$$

The fractional saturation of the A site, n_A, for a given tRNA concentration, c_{T^0}, is obtained from

$$n_A(c_{T^0}) = \{N - [N^2 - 4c_{TA}(c_{T^0})c_{R^0}]^{1/2}\}/(2c_{R^0}) \quad (2c)$$

with

$$N = c_{TA}(c_{T^0}) + c_{R^0} + 1/K_A$$

In analogy to the treatment of P site binding (see eq 3a and 2b), the concentration of A sites, c_{UA}, which at a given tRNA concentration, c_{T^0}, remain unoccupied and the fractional saturation of A sites with the indicator, the ternary complex Phe-$tRNA^{Phe}$·EF-Tu·GTP, n_{IA}, are given by eq 3b and 2d:

$$c_{UA}(c_{T^0}) = [1 - n_A(c_{T^0})]c_{R^0} \quad (3b)$$

$$n_{IA}(c_{T^0}) = \{Q - [Q^2 - 4c_{UA}(c_{T^0})c_{I^0}]^{1/2}\}/(2c_{I^0}) \quad (2d)$$

with

$$Q = c_{I^0} + c_{UA}(c_{T^0}) + 1/K_{IA}$$

Finally, as discussed above for the titration of the P site (cf. eq 4a), n_{IA} is related to the amount of bound indicator tRNA, I_A, according to

$$I_A(c_{T^0}) = I_{max}n_{IA}(c_{T^0}) + I_B \quad (4b)$$

Fitting of eq 4b to the titration data (see above) yields the concentration of ribosomes being active in tRNA binding to the A site. In addition to the binding constants of the P site (see above), the following constants, K_A, of 1.5×10^7 and 3.3×10^7 M^{-1} for deacylated and N-AcPhe-$tRNA^{Phe}$, respectively, were taken for A site binding in buffer A; in buffer B at 10 mM Mg^{2+}, the respective binding constants are 5.0×10^6 and 2.0×10^7 M^{-1}. A binding constant for the ternary complex, K_{IA}, of 1.0×10^9 M^{-1} was used for both ionic conditions.

The fitting procedure described above yields optimal results when both the first tRNA added and the indicator tRNA are strongly bound to the inspected site, i.e., $c_{R^0} >> 1/K$, and when the indicator is added in small amounts relative to the ribosomes. It should be mentioned that under conditions of nonstoichiometric binding, i.e., $c_{R^0} \approx 1/K$, this type of titration can be used for the determination of binding constants. In this case, strong binding of the indicator tRNA is particularly advantageous.

Results

Ribosome Activity and tRNA Binding Sites. The determination of the number of tRNA molecules bound to a ribosome of course requires knowledge of the concentration of active macromolecules ("activities"). The fact that the tRNA binding capacity of ribosome preparations may vary poses a major difficulty here, since it precludes the exact determination of the concentration of active ribosomes by absorption measurements.

In order to obtain an easily accessible measure for ribosome activity, we took advantage of the fact that upon titrating poly(U)-programmed *E. coli* ribosomes with $tRNA^{Phe}$, the sites are occupied in a sequential fashion, the P site being occupied first (Watanabe, 1972). The decrease of the number of unoccupied P sites during the titration then can be determined from the puromycin reactivity of N-AcPhe-$tRNA^{Phe}$ added in a subsequent incubation step ("indicator reaction"). In an analogous manner, unoccupied A sites can be measured by using the ternary complex Phe-$tRNA^{Phe}$·EF-Tu·GTP, which specifically binds to the A site, as an indicator.

Of course, the determination of unoccupied sites by subsequent indicator reactions requires that the rate of exchange from the occupied sites is slow relative to the time needed for binding the indicator tRNA. The stability of tRNA bound to P and A sites was tested with ^{32}P-labeled $tRNA^{Phe}$ and N-AcPhe-$tRNA^{Phe}$ by using both dilution jump and chase experiments. The half-lives of deacylated $tRNA^{Phe}$ at 20 (10) mM Mg^{2+} concentration were found to be >10 (8) h for the P site and 2 (1) h for the A site. Similar numbers, although reversed for P and A sites, were found for N-AcPhe-$tRNA^{Phe}$.

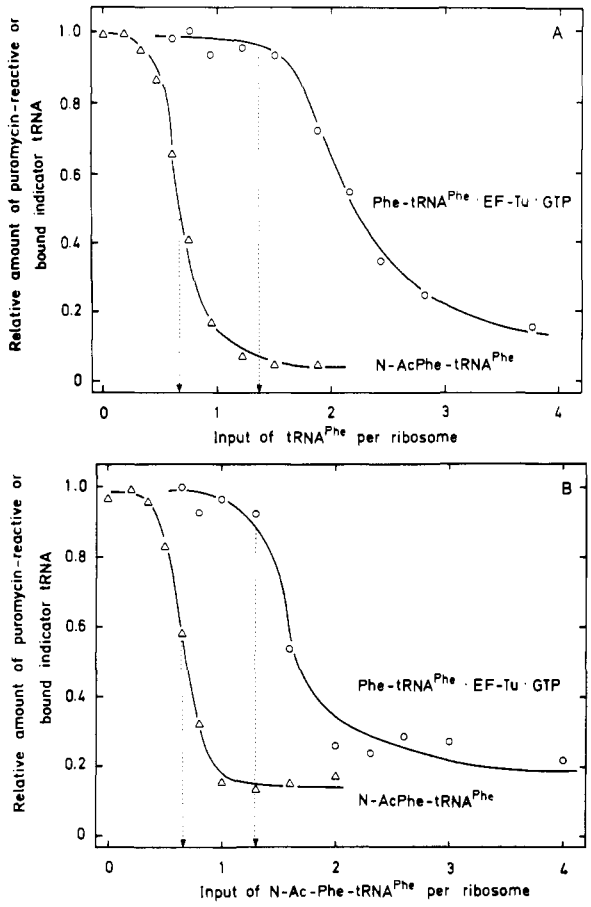

FIGURE 1: Indicator titrations of the ribosomal P and A sites. Increasing amounts of either (A) deacylated tRNA^{Phe} or (B) *N*-Ac-Phe-tRNA^{Phe} (specific radioactivity 10 Ci/mol) from yeast were incubated with ribosomes in buffer A, and the indicator reactions were performed with *N*-AcPhe-tRNA^{Phe} (504 Ci/mol) for the P site and Phe-tRNA^{Phe}·EF-Tu-GTP for the A site as described under Materials and Methods. The solid lines represent the curves obtained by fitting the data to the models for the titration of the P or the A site as described under Materials and Methods; the resulting fractions of active P sites (0.65) and P plus A sites (1.3) are indicated by dotted lines. To normalize the ordinate for both assays, the original dpm values were divided by the value obtained for completely bound indicator tRNA.

The lowest stability, with a half-life of about 10 min, was found for A site bound deacylated tRNA^{Phe} in the presence of ternary complex. Thus, under all conditions examined the amount of tRNA prebound to the P and A sites will not change significantly during the indicator experiments.

Figure 1A shows a titration experiment in which poly(U)-programmed ribosomes were first incubated with increasing amounts of yeast tRNA^{Phe} and then with either *N*-AcPhe-tRNA^{Phe} or the ternary complex as indicators. The concentration of ribosomes (0.5 μM) was well above the dissociation constants of the P and A site complexes of tRNA^{Phe} (see Materials and Methods), thus assuring the nearly stoichiometric uptake of the added tRNA by the ribosomes. As detailed in the theoretical section under Materials and Methods, this condition is optimal for the determination of ribosome activity examined here. It is clearly seen from the indicator reactions that the P site is occupied first and that tRNA binding to the A site becomes significant only after the P site has been filled. From the sigmoidal titration curves, the concentration of ribosomes which are active in binding tRNA^{Phe} to both P and A sites was estimated by computer fitting, as detailed under Materials and Methods. The analysis revealed that the P site was filled with a stoichiometry of 0.65

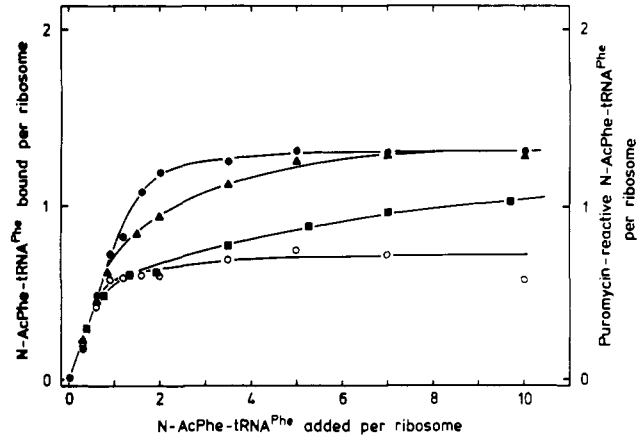

FIGURE 2: Titrations of ribosomes with *N*-AcPhe-tRNA^{Phe}. The titrations were performed at Mg^{2+} concentrations of 20 mM (buffer A) (●, ○), 10 mM (buffer B) (▲), and 6 mM (buffer B) (■), and the nitrocellulose filtration (closed symbols) and puromycin (open symbols) assays were carried out as described under Materials and Methods.

tRNA^{Phe} molecule per ribosome. The same stoichiometry was obtained for the subsequent A site binding. Apparently, 65% of the ribosomes were active in binding tRNA^{Phe} to both P and A sites.

Essentially the same result was obtained when the ribosomes were titrated with *N*-Ac[^{14}C]Phe-tRNA^{Phe} from yeast (and *E. coli*, not shown) of low specific radioactivity instead of deacylated tRNA^{Phe}, and the subsequent indicator reactions were performed with *N*-Ac[^{14}C]Phe-tRNA^{Phe} or [^{14}C]Phe-tRNA^{Phe} of 50 times higher specific radioactivity (Figure 1B). Comparable results, i.e., total binding of about 1.3 molecules of *N*-AcPhe-tRNA^{Phe} per ribosome, were also obtained in the saturation titration experiments shown below (Figure 2).

In order to further characterize the ribosomes, we measured polyphenylalanine synthesis. In the chain termination assay with ^{3}H-labeled puromycin (Chinali & Parmeggiani, 1980), the ribosomes exhibited an activity of 68%, similar to the activity in the binding experiments. Finally, the initial rate of polyphenylalanine synthesis was measured in the system of Wagner et al. (1982) and was found to be around 8 s^{-1}, not far from the in vivo rate.

Two Molecules of N-AcPhe-tRNA^{Phe} Are Bound per Active Ribosome. In saturation titration experiments at Mg^{2+} concentrations of 20 and 10 mM, 1 mol of poly(U)-programmed ribosomes bound 1.3 mol of *N*-AcPhe-tRNA^{Phe} (Figure 2). According to the puromycin reactivity, the ribosome-bound *N*-AcPhe-tRNA^{Phe} at saturation was equally distributed between P and A sites. We conclude that about 65% of the ribosomes bound *N*-AcPhe-tRNA^{Phe} to both P and A sites simultaneously. *N*-AcPhe-tRNA^{Phe} from *E. coli* yielded the same results (data not shown). (It should be mentioned that, in the presence of an excess of *N*-AcPhe-tRNA^{Phe}, the puromycin assay may no longer be specific for P site bound *N*-AcPhe-tRNA^{Phe}, thus leading to an overestimation of the occupancy of the P site; this problem is discussed in more detail below.) Qualitatively similar binding results were obtained at 6 mM Mg^{2+} (Figure 2), although binding of the second molecule of *N*-AcPhe-tRNA^{Phe}, i.e., A site binding, under these conditions apparently was too weak to reach saturation even at a 10-fold excess of *N*-AcPhe-tRNA^{Phe}.

For a quantitative evaluation of the binding curves of Figure 2 with respect to binding stoichiometry, it is important to use highly charged *N*-AcPhe-tRNA^{Phe} (at least 85%, 100% being 1750 pmol/A_{260} unit). Otherwise, the apparent stoichiometry reflects not only the binding activity of the ribosomes but also

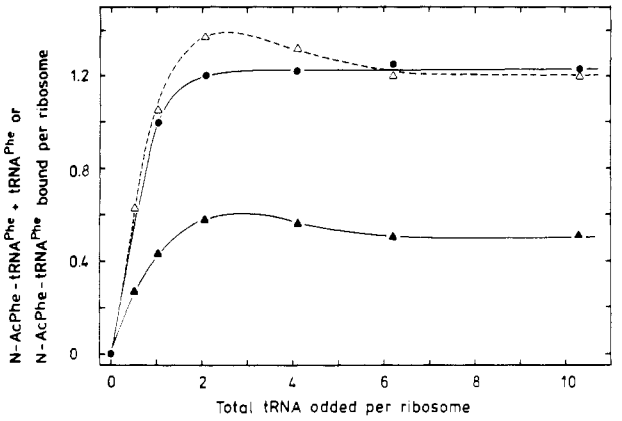

FIGURE 3: Influence of the charging level of $tRNA^{Phe}$ on the extent of ribosome binding of *N*-AcPhe-$tRNA^{Phe}$. The titrations were carried out in buffer A with *N*-AcPhe-$tRNA^{Phe}$ containing either 5% (●) or 58% (▲) deacylated $tRNA^{Phe}$. The data points represented by open symbols were calculated from the latter points by taking into account the charging level.

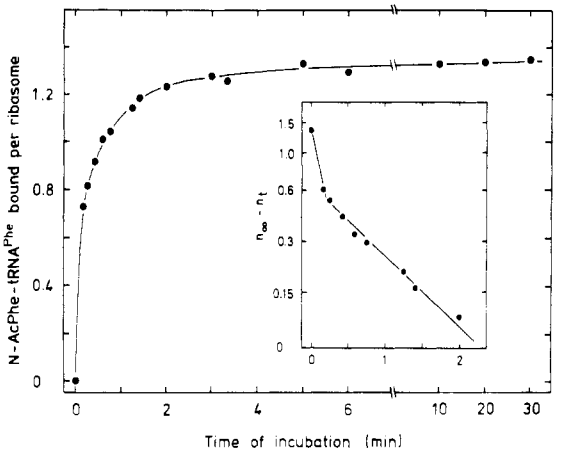

FIGURE 4: Time course of *N*-AcPhe-$tRNA^{Phe}$ binding to the ribosomal P and A sites. A 4-fold excess of *N*-AcPhe-$tRNA^{Phe}$ was added to ribosomes (buffer A) and the amount of ribosome-bound material determined at the times indicated. The inset shows a semilogarithmic plot of the data, where n_t and n_∞ represent *N*-AcPhe-$tRNA^{Phe}$ bound per ribosome at time t and at saturation, respectively.

the charging level of the tRNA. Furthermore, in the presence of a significant amount of deacylated $tRNA^{Phe}$, it is not sufficient to take the total concentration of charged plus uncharged tRNA for the calculation. In addition, the saturation value has to be determined at a sufficiently high excess of *N*-AcPhe-$tRNA^{Phe}$, since the specificity of the additional (E) site for deacylated tRNA (see below) leads to an artificially high amount of P and A site bound *N*-AcPhe-$tRNA^{Phe}$ in the early points of the titration. This is demonstrated by the experiment shown in Figure 3, in which a mixture of 42% *N*-AcPhe-$tRNA^{Phe}$ and 58% deacylated $tRNA^{Phe}$ was used for the titration. In this case, the true saturation level is only reached at a 6-fold excess, and the stoichiometry has to be calculated on the basis of total added tRNA, in order to come to the value of about 1.3 obtained from the data of Figure 2.

The conclusion that the saturation curves shown in Figure 2 represent the binding of *N*-AcPhe-$tRNA^{Phe}$ to two different ribosomal sites, P and A, is also supported by the biphasic time course of binding, which was observed at Mg^{2+} concentrations between 20 (Figure 4) and 10 mM (not shown). In accordance with previous kinetic data (Wintermeyer & Robertson, 1982), the rapid binding of *N*-AcPhe-$tRNA^{Phe}$ to the P site is followed by the slow binding of a second molecule of *N*-AcPhe-$tRNA^{Phe}$ to the A site.

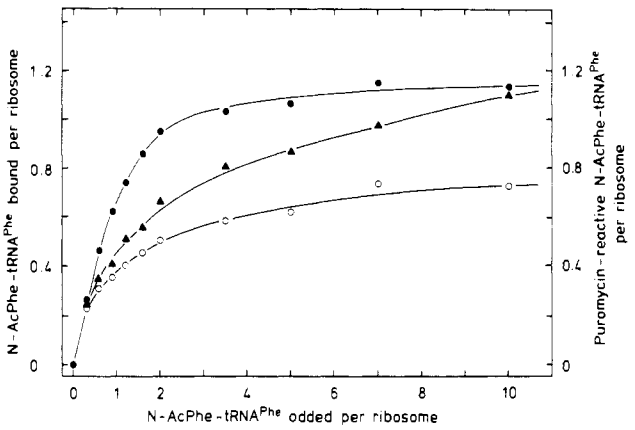

FIGURE 5: Titrations of low-salt ribosomes with *N*-AcPhe-$tRNA^{Phe}$. Titrations were carried out at Mg^{2+} concentrations of 20 mM (buffer A) (●, ○) and 10 mM (buffer B) (▲) and analyzed by the nitrocellulose (closed symbols) and puromycin (open symbols) assays.

The present results are at variance with a recent report of Rheinberger et al. (1981). From the observation that their ribosomes bound *N*-AcPhe-$tRNA^{Phe}$ at a 1:1 stoichiometry with all of it in a puromycin-reactive state, these authors concluded that their ribosomes were 100% active and that binding of one molecule of *N*-AcPhe-$tRNA^{Phe}$ to the P site excluded further binding of *N*-AcPhe-$tRNA^{Phe}$ to the A site.

For a direct comparison, we repeated the titration experiment of Figure 2 with ribosomes prepared according to the protocol of Rheinberger & Nierhaus (1980), which uses a low salt concentration (0.03 M ammonium chloride) throughout the preparation. As shown in Figure 5, these ribosomes also bound significantly more than one molecule of *N*-AcPhe-$tRNA^{Phe}$, about 1.1 mol/mol, at both 10 and 20 mM Mg^{2+}, indicating binding to two ribosomal sites of 55% activity each. This contention is supported by the observation of a biphasic time course of complex formation (15 mM Mg^{2+}, not shown), as seen with our ribosomes (cf. Figure 4). Final proof was obtained by using the indicator titrations, which confirmed the activity of 55% for the low-salt ribosomes (data not shown).

As mentioned above, in the presence of an excess of *N*-AcPhe-$tRNA^{Phe}$ the puromycin assay tends to an "overreaction". This is seen in Figure 5, where the puromycin reactivity at the plateau exceeds 50% of the bound material. Occasionally, such a behavior was also observed with our standard ribosomes, particularly at Mg^{2+} concentrations of 10 mM or below. In fact, under these conditions, the puromycin reaction does not halt after the P site bound *N*-AcPhe-$tRNA^{Phe}$ has reacted but continues at a slower rate until eventually all bound material, or even more, has reacted.

The molecular basis for the overreaction remains unclear to us. For instance, the dissociation of deacylated $tRNA^{Phe}$ from the P site, also under the conditions of the puromycin reaction, is much too slow (R. Lill, unpublished results) to account for either additional binding of free *N*-AcPhe-$tRNA^{Phe}$ to the P site or nonenzymatic translocation of *N*-AcPhe-$tRNA^{Phe}$ from the A site. In any case, the results show that the puromycin reactivity alone must not be taken as prima facie evidence for P site location, whenever *N*-AcPhe-$tRNA^{Phe}$ is present in excess over ribosomes. The problems with the puromycin reaction may explain, at least in part, the discrepancies between the present results and those of Rheinberger et al. (1981).

Three Ribosomal Sites for Deacylated $tRNA^{Phe}$. In order to determine the number of ribosomal binding sites for deacylated tRNA, we performed titration experiments with ^{32}P-labeled $tRNA^{Phe}$ from both yeast and *E. coli*. In addition, the

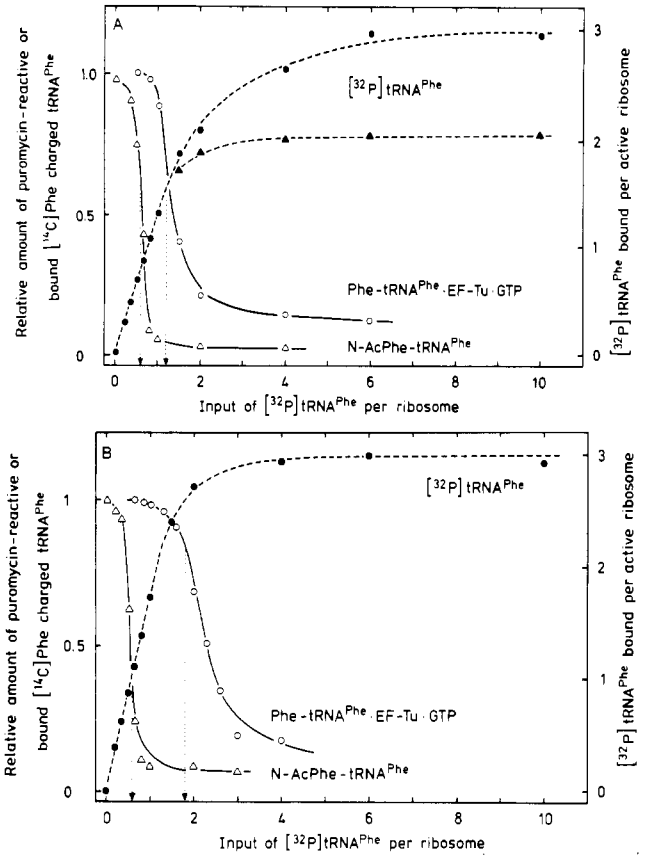

FIGURE 6: Ribosome binding of deacylated tRNA^{Phe} from (A) yeast and (B) *E. coli*. Titrations were performed in buffer A with ^{32}P-labeled tRNA^{Phe} and the complexes analyzed by nitrocellulose filtration without (●) or with (▲) prior dilution. The indicator reactions for the determination of unoccupied P and A sites were carried out and evaluated by computer fitting (solid lines) as described under Materials and Methods. Bound ^{32}P-labeled tRNA^{Phe} is given relative to the fraction of active ribosomes, as determined by the indicator reactions (dotted lines).

indicator reaction was utilized to determine the activity of the ribosomes in the actual experiment. Figure 6A,B shows the binding curves obtained at 20 mM Mg^{2+}; very similar curves were observed at 15 mM Mg^{2+}. The ordinate values of Figure 6 were calculated on the basis of the activity of the ribosomes as determined by the indicator reactions shown in the figure. At the plateau, about 1.9 mol of ^{32}P-labeled tRNA^{Phe} was bound per mol of total ribosomes, corresponding to the binding of three molecules of deacylated tRNA^{Phe} per active ribosome. Thus, in accordance with the reports of Rheinberger et al. (1981, 1983), we also observe the third binding site (E site) in addition to P and A sites, which is accessible for deacylated tRNA only.

From the indicator reactions with ternary complex, shown in Figure 6, an interesting difference between the tRNA^{Phe}s from yeast and *E. coli* becomes apparent. In contrast to the former, which bound to the additional site last, the latter bound to this site prior to or concomitantly with the A site. This follows from the observation that the A site is filled with an apparent stoichiometry of 1.9 tRNA molecules per ribosome, i.e., 3 tRNAs per active ribosome; models which assumed the A site to be filled as the second site led to inconsistent results. The different sequence of site occupation is due to the fact that the heterologous tRNA is bound to the E site with a lower stability. This is also reflected in the observation that binding of the third molecule of yeast tRNA^{Phe} is detectable in the filtration assay only when the reaction mixture is spotted onto the filter directly, without prior dilution, indicating a rather

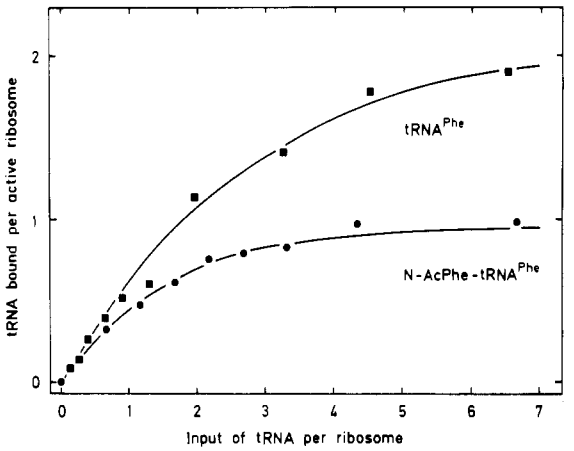

FIGURE 7: Ribosome binding of *N*-AcPhe-tRNA^{Phe} (●) and of *E. coli* [^{32}P]tRNA^{Phe} (■) in the absence of mRNA. Titrations were performed in buffer A as described in Figures 2 and 6, except that no poly(U) was present.

rapid dissociation (Figure 6A). Indeed, direct kinetic experiments yield a dissociation rate constant around 0.1 s^{-1} (W. Wintermeyer, unpublished results). *E. coli* tRNA^{Phe}, on the other hand, is bound more stably, since its binding could also be detected by filtration with prior dilution, at least at Mg^{2+} concentrations of 15 mM and above. It should be stressed, however, that the limited stability of the E site complexes cannot be compared to that of the P and A site complexes, which are extremely stable (half-lives of hours).

Furthermore, the stability of the E site complexes was found to be strongly dependent upon the Mg^{2+} concentration. Thus, the binding of yeast tRNA^{Phe} to the E site was no longer detectable at Mg^{2+} concentrations of 10 mM and below, at least not by the filtration assay (data not shown), whereas binding to both P and A sites was still observed, as judged from the indicator reactions. Similarly, at 10 mM Mg^{2+}, binding of *E. coli* tRNA^{Phe} to the E site was not complete even at tRNA to ribosome ratios greater than 10, indicating a rather low affinity.

Binding of tRNA^{Phe} to the E Site Does Not Require Poly(U). Upon omission of poly(U), the ribosomes bind only one molecule of *N*-AcPhe-tRNA^{Phe} (Figure 7). From the puromycin reactivity, it is clear that binding takes place in the P site exclusively. The A site, apparently, is not occupied in the absence of mRNA. Thus, the observed binding of two molecules of deacylated tRNA^{Phe} per active ribosome (Figure 7) suggests that, in addition to the P site, the E site is also occupied in the absence of mRNA. This result is at variance with the report of Rheinberger et al. (1981) and supports the results of Grajevskaja et al. (1982). Most likely, the discrepancy is due to the low stability of the E site complex, necessitating rapid filtration in order to be detectable.

Discussion

Ribosome Activity and tRNA Binding Sites. The crucial point in determining the number of ribosomal tRNA binding sites is the characterization of the ribosomes with respect to tRNA binding activity. This requires that the site location of the tRNA is known with certainty. Generally, P site bound *N*-AcPhe-tRNA^{Phe} is determined by the puromycin assay. The present results, however, demonstrate that in the presence of an excess of *N*-AcPhe-tRNA^{Phe}, this assay may lead to erroneous conclusions regarding the proportion of ribosome-bound *N*-AcPhe-tRNA^{Phe} being bound to the P site and, with that, the tRNA binding activity of the ribosomes. This potential error can be avoided by using an indirect assay, which is based

on the puromycin reactivity of a small amount of *N*-AcPhe-tRNA^{Phe} added after the binding of the tRNA species under study is completed. The puromycin reactivity observed in the indicator reaction, which is a measure for P sites remaining unoccupied in the first incubation, is taken to determine the fraction of active P sites. As described under Results, the fraction of active A sites can be determined in an analogous fashion by using the ternary complex Phe-tRNA^{Phe}·EF-Tu·GTP as an indicator for unoccupied A sites. For a given ribosome preparation, the two sites were always found to be equally active.

The activity of the tight-couple ribosomes prepared according to our standard procedure (Robertson & Wintermeyer, 1981), as determined by the assays outlined above, in several preparations varied between 60% and 80%. Similar figures were reported by a number of other groups (Odinzov & Kirillov, 1978; Chinali & Parmeggiani, 1980; Jelenc, 1980) and a somewhat higher activity (90%) by Schmitt et al. (1981). Almost full activity, 90–100%, has been claimed by Rheinberger et al. (1981). However, this figure rests on the assumption of an exclusion principle, i.e., that binding of *N*-AcPhe-tRNA^{Phe} to the P site prevents its further binding to the A site, which was postulated on the basis of the observation that in the presence of saturating amounts of *N*-AcPhe-tRNA^{Phe} all ribosome-bound material was puromycin reactive. As shown under Results and discussed in the preceding paragraph, this behavior is due to an overreaction of excess *N*-AcPhe-tRNA^{Phe} and must not be interpreted as to suggest an exclusive, highly efficient P site binding.

As to the binding of deacylated tRNA^{Phe}, we find three molecules bound per ribosome. On the basis of the present data, we can exclude the existence of other nonoverlapping binding sites stronger than 2×10^5 M^{-1} and stable enough to be detectable by the filtration assay. Our results confirm the existence of an additional, third tRNA binding site of *E. coli* ribosomes, being accessible only for deacylated tRNA, as reported by Rheinberger et al. (1981). It should be noted, however, that these authors calculated the stoichiometry of 3 on the basis of 100% activity of the ribosomes, which was shown above to be incorrect. In our case, the stoichiometry of 3 is obtained by taking into account the fractional activity (65%) of the ribosomes. Only then can we arrive at a consistent picture: three molecules of deacylated tRNA^{Phe} and two molecules of *N*-AcPhe-tRNA^{Phe} are bound per active ribosome.

Possible Function of the Third tRNA Binding Site. Following a proposal of Noll (1966), the third site has been discussed to represent an exit site, which during translocation receives the deacylated tRNA from the P site (Rheinberger et al., 1983). What properties can be expected for such a site? On the one hand, an additional tRNA binding site could help in the release of the tRNA by dividing the activation energy needed for the dissociation from the ribosome. Such an exit function would imply a lower tRNA binding affinity for the E site relative to the P site. On the other hand, it has been postulated that the tRNA, after leaving the P site, is stably bound to the E site, because a second tRNA, in addition to the P site bound tRNA, is required to keep the mRNA in a fixed position on the ribosome (Rheinberger & Nierhaus, 1983). In addition, the same authors proposed that tRNA release from the E site is triggered by occupation of the A site, which was thought to lower the affinity of the E site. For an evaluation of these proposals, tRNA binding to the E site has to be considered according to the following three criteria: (1) stability at physiological conditions; (2) influence of the mRNA; and (3) coupling between A and E sites.

As to the first point, the present results show that the stability of the E site complex is low as compared to the other two ribosomal sites under any experimental condition we have tested. This is particularly true for low Mg^{2+} concentrations. Furthermore, in translocation experiments at 10 mM Mg^{2+}, about half of the P site bound tRNA was immediately released from the ribosome upon addition of EF-G and GTP (J. M. Robertson, unpublished results). Thus, at physiological conditions, the population of the E site after translocation probably is very low.

Second, in accordance with the report of Grajevskaja et al. (1982), we find that tRNA binding to the E site does not depend upon the presence of mRNA. Conversely, the tRNA in the E site will not be of great help in stabilizing the mRNA on the ribosome.

Finally, translocation experiments clearly show that E site bound tRNA^{Phe} is not released upon binding of Phe-tRNA to the A site but is rapidly chased upon adding excess deacylated tRNA (J. M. Robertson, unpublished results). These findings do not support a model (Rheinberger & Nierhaus, 1983) in which A and E sites are coupled in the sense that occupation of one site weakens tRNA binding to the other.

To summarize, the present results suggest an exit function of the additional tRNA binding site, in the sense that, during translocation, the tRNA is transferred from the P to the E site, where it transiently binds before leaving the ribosome. Most likely, the codon–anticodon interaction is disrupted upon the transfer to the E site.

Different E Site Binding of Homologous and Heterologous tRNA. The observation that the tRNA^{Phe} from *E. coli* binds to the E site with a significantly higher stability than tRNA^{Phe} from yeast was unexpected, since the tRNAs from the two organisms work equally well in polyphenylalanine synthesis with *E. coli* ribosomes. Thus, the observed difference in E site binding may not be important. On the other hand, as far as we can see, it demonstrates for the first time that the *E. coli* ribosome is able to distinguish tRNAs on a basis other than codon–anticodon interaction. It will be interesting now to see whether the difference is due to specific modified nucleosides and whether closer inspection of the other two ribosomal sites, for which up to now no difference in the binding of homologous and heterologous tRNAs has been reported, will reveal differences too.

Acknowledgments

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Registry No. GTP, 86-01-1.

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^{31}P and Two-Dimensional $^{31}P/^{1}H$ Correlated NMR Spectra of Duplex d(Ap[^{17}O]Gp[^{18}O]Cp[^{16}O]T) and Assignment of ^{31}P Signals in d(ApGpCpT)$_2$–Actinomycin D Complex†

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ABSTRACT: A solid-phase phosphoramidite method was used for the synthesis of unlabeled and phosphoryl-labeled d(Ap[^{17}O]Gp[^{18}O]Cp[^{16}O]T). The ability to label the phosphoryl oxygens of d(ApGpCpT) and thus assign the ^{31}P signals, combined with a two-dimensional $^{31}P/^{1}H$ chemical shift correlated NMR spectral technique, provided a novel means for the ready assignment of the H5′ and H3′ protons coupled to the phosphates. Phosphoryl labeling has also allowed us to assign the ^{31}P NMR signals in the actinomycin D–d(Ap[^{17}O]Gp[^{18}O]Cp[^{16}O]T)$_2$ duplex complex and confirm that the drug intercalates between the GpC stacked base pairs.

The interaction of drugs such as the antitumor antibiotic actinomycin D (Act D)[1] with nucleic acids is believed to perturb the conformation of the sugar–phosphate backbone. Indeed, much of the pharmacological activity of this and related drugs derives from their direct intercalation between stacked base pairs of the nucleic acids (Lown, 1977). When DNA structure is perturbed, these drugs may inhibit the synthesis of nucleic acids and interfere with cellular mitosis.

^{31}P NMR has been particularly useful in defining the intercalative mode of interaction (Muller & Crothers, 1968) of drugs such as actinomycin D with nucleic acids (Gorenstein & Goldfield 1984), since ^{31}P NMR chemical shifts in nucleic acids are a sensitive probe of P–O ester bond torsional angles (Gorenstein & Kar, 1975; Gorenstein, 1978, 1981, 1984). In one of the earliest demonstrations of ^{31}P spectral perturbations on drug binding, Patel (1974, 1976) and Reinhardt & Krugh (1977) showed that actinomycin D (Act D) shifted several phosphate diester signals up to 2.6 ppm downfield from the double-helical signals on binding to oligonucleotides containing dG-C base pairs. Thus, Patel (1976) showed that new ^{31}P signals appeared 1.6 and 2.6 ppm downfield in a d(CGCG)·Act D (2:1) complex.

These shifts are consistent with the Jain & Sobell (1972) model for these intercalated complexes: partial unwinding of a specific section of the double helix allows these planar heterocyclic drugs such as Act D to stack between two base pairs. X-ray studies of various intercalating drug–duplex complexes (Reddy et al., 1979; Shieh et al., 1980) suggest that the major backbone deformation of the nucleic acid on intercalation of the drug involves the C-5′–O-5′ torsional angle. However, in several complexes (Reddy et al., 1979) the P–O ester torsional angles (ω, ω') are altered from the normal B DNA ω, ω' conformation values of 290°, 290° (g^-,g^-; –gauche, –gauche) to values such as 273°, 323° and even 289.6°, 204.8° ($\sim g^-$,t) (Bhandary et al., 1984). Deshielding of the ^{31}P signals of up to 2.0 ppm has been calculated (Gorenstein & Kar, 1975; Gorenstein, 1983a,b, 1984) for torsional angle changes of this magnitude.

Since Act D is known to have a preference for G-C-rich DNA, it has been assumed that the site of intercalation is between two stacked d(G-C) base pairs (Remers, 1978). Unfortunately the one authenic X-ray structure of a Act D·d(G-C) complex (Takusagawa et al., 1982) does not contain

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[1] Abbreviations: Act D, actinomycin D; COSY, ^{1}H two-dimensional J-correlation NMR; NOESY, ^{1}H two-dimensional NOE correlation NMR; EDTA, (ethylenedinitrilo)tetraacetic acid; FID, free induction decay; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; NOE, nuclear Overhauser effect; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; 2-D, two dimensional.

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