Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273-335.
Vallee, B. L., Williams, R. J. P., & Hoch, F. L. (1959) *J. Biol. Chem. 234*, 2621-2626.
Van Holde, K. E., & Baldwin, R. L. (1958) *J. Phys. Chem. 62*, 734-743.
Von Wartburg, J.-P., Bethune, J. L., & Vallee, B. L. (1964) *Biochemistry 3*, 1775-1782.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406-4412.
Yphantis, D. A. (1964) *Biochemistry 3*, 297-317.

Detection of Ligand-Induced Conformational Changes in Phenylalanyl-tRNA Synthetase of *Escherichia coli* K10 by Laser Light Scattering†

Eggehard Holler,* Chun-Chen Wang, and Norman C. Ford, Jr.

ABSTRACT: The diffusion constant of phenylalanyl-tRNA synthetase has been measured by laser light scattering under conditions of complex formation with Mg^{2+}, L-phenylalanine, MgATP, $tRNA^{Phe}$, modified $tRNA^{Phe}$, $tRNA^{Phe}$ (yeast), and noncognate tRNA. The diffusion constant (pH 7.5, 20 °C) of the free enzyme is $(2.85 \pm 0.005) \times 10^{-7}$ cm^2 s^{-1}, of the enzyme·Mg^{2+} complex $(2.40 \pm 0.05) \times 10^{-7}$ cm^2 s^{-1}, and of the enzyme·Mg^{2+}·$tRNA^{Phe}$ complex $(2.95 \pm 0.06) \times 10^{-7}$ cm^2 s^{-1}. The effect of $tRNA^{Phe}$ is only seen when the enzyme is saturated with Mg^{2+}. The smaller substrates exhibit no effect besides a small increase of the value of the diffusion constant under conditions where the enzyme·phenylalanyladenylate is synthesized. Of the noncognate $tRNA^{Tyr}$ and $tRNA^{Ile}$, the latter is able to associate with the enzyme, causing the value of the diffusion constant to increase. $tRNA^{Phe}$ (yeast) and $tRNA^{Phe}_{h\nu}$ (photo-cross-linked $tRNA^{Phe}$) exhibit similar effects. The observed variation of the diffusion constant is attributed to conformational changes of the enzyme. The opposite effects of Mg^{2+} and $tRNA^{Phe}$ are interpreted as an expansion and recontraction, respectively, of the enzyme molecule. In several cases, the effects were used to follow a titration of the enzyme with a ligand. Dissociation constants were calculated from the resulting titration curves, yielding values which are in agreement with those obtained by other techniques. It is established by comparison that of the two possible binding sites for each Mg^{2+} and $tRNA^{Phe}$ the diffusion constant reflects occupation of only a single class of sites.

One of the important early steps in protein synthesis is the formation of aminoacyl-tRNA. This process involves two steps, both mediated by a tRNA synthetase specific to a given tRNA–amino acid pair. The first step is the chemical activation of the amino acid, and the second, the attachment of the amino acid to the tRNA.

The second step is commonly believed to be accompanied by one or more changes in the conformation of the enzyme. Indeed, there is a substantial body of literature reporting conformation changes upon binding of tRNA as detected by several techniques. For example, changes in conformation of the enzyme–substrate complex were detected by using kinetic studies of the yeast Phe, *Escherichia coli* Tyr, and yeast Ser systems (Riesner et al., 1976; Krauss et al., 1976; Rigler et al., 1976), circular dichroism measurements of the yeast Tyr system (Ohta et al., 1967), X-ray small-angle scattering of the yeast Phe system (Pilz et al., 1979), and neutron-scattering studies of the *Escherichia coli* Met (Dessen et al., 1978) and yeast Glu (Zaccai et al., 1979) enzymes. Changes in the tRNA conformation upon binding the enzyme have been studied in the *E. coli* Glu (Willick & Kay, 1976) and *E. coli* Phe (Favre et al., 1979) systems by using circular dichroism and in the yeast Phe system by using fluorescence studies of the wybutine base (Krauss et al., 1976).

In spite of this work, a number of questions remain unanswered. Among these are the following: (1) Does the conformation rearrangement of a synthetase·tRNA complex involve changes in both the enzyme and the tRNA or only in the tRNA? (2) It is known that Mg^{2+} has profound effects on reactions catalyzed by phenylalanyl-tRNA synthetase of *E. coli* and on the formation of the enzyme·Phe-$tRNA^{Phe}$ complex (Bartmann et al., 1975a; Holler, 1976). Are these effects caused by or, at least, accompanied by conformation changes? (3) Anticooperative binding of certain substrates has been observed for aminoacyl-tRNA synthetases (Dessen et al., 1978, and references contained therein) and for the Phe-specific enzyme of *E. coli*, L-phenylalanine, and $tRNA^{Phe}$ (Bartmann et al., 1975a). It is of interest to know if the structural changes in the enzyme accompanying the binding of phenylalanine and $tRNA^{Phe}$ are similar. (4) It has been suggested that conformation changes provide a mechanism for the differentiation between cognate and noncognate tRNAs which also bind to the synthetase (Krauss et al., 1976; Rigler et al., 1976). Can the proposed changes be detected by their effect on the hydrodynamic properties of the enzyme as measured by the light-scattering technique?

In the present studies we have employed the laser light-scattering technique to study the diffusion constant of the enzyme *E. coli* phenylalanyl-tRNA synthetase and its complexes with Mg^{2+}, cognate, modified cognate, and noncognate tRNAs and with small substrates L-phenylalanine and MgATP. It provides an ideal tool for studying the questions outlined above. Several factors contribute to this choice of technique in contrast to techniques employed earlier. For example, in

† From the Fachbereich Biologie und Vorklinikum, Universität Regensburg, D-8400 Regensburg, Federal Republic of Germany (E.H.), and the Department of Physics, University of Massachusetts, Amherst, Massachusetts 01002 (C.-C.W. and N.C.F.). *Received May 7, 1980.* This work was generously supported in the form of a travel grant to E.H. by the Deutsche Forschungsgemeinschaft.

0006-2960/81/0420-0861$01.00/0

contrast to neutron-scattering studies, the data are obtained rapidly so that it is practical to study the effects of a variety of solution conditions and substrates. Further, unlike techniques such as circular dichroism which detects changes in helical content of the entire complex, the laser light-scattering method probes the overall shape of the complex and therefore is most sensitive to changes in conformation of the enzyme itself. This is because the substrates are all sufficiently small that their addition to the enzyme without an accompanying change in conformation would not perturb the hydrodynamic properties to a significant extent. Thus it is possible to monitor conformational changes which might otherwise not affect optical properties (absorbance, fluorescence, circular dichroism, etc.). Large molecules such as phenylalanyl-tRNA synthetase (M_w = 267 000) can be investigated at concentrations that would be inpractically low for absorbance measurements.

Materials and Methods

Samples. L-Phenylalanyl-tRNA synthetase (EC 6.1.1.20) was isolated from *Escherichia coli* K10 in the presence of phenlymethanesulfonyl fluoride by using the method of Kosakowski & Böck (1970). The preparation had a specific enzyme activity of 54 000 nmol mg^{-1} h^{-1} and 1.9 mol of active sites/mol of enzyme (Bartmann et al., 1975b). Alkaline phosphatase (EC 3.1.3.1) and inorganic pyrophosphatase (~200 U/mg) (EC 3.6.1.1) were obtained from Boehringer (Mannheim). $tRNA^{Phe}$ (1400 pmol of L-phenylalanine per A_{260} unit in water) of *E. coli* MRE 600 and brewer's yeast were purchased from Boehringer (Mannheim). $tRNA^{Ile}$ (1300 pmol of L-isoleucine and less than 4 pmol of L-phenylalanine per A_{260} unit in water) and $tRNA^{Tyr}$ (540 pmol of L-tyrosine and less than 4 pmol of L-phenylalanine per A_{260} unit in water) were prepared from unfractionated tRNA (Zubay, 1962) of *E. coli* K10 by using chromatography on benzoylated DEAE-cellulose (Gillam et al., 1967) and RPC-5 columns (Pearson et al., 1971). $tRNA^{Phe}$-CCp[1] and $tRNA^{Phe}$-CC were prepared by periodate treatment in the presence of lysine to eliminate the 3′-terminal adenosine and by removal of the 3′-terminal phosphate in the presence of alkaline phosphatase (Khym & Uziel, 1968). Seven percent of the acceptance of L-phenylalanine persisted throughout the oxidation by periodate. $tRNA^{Phe}_{hv}$ was prepared by UV-induced cross-linking of the 4-thiouridine in position 8 to the cytidine in position 13 (Favre & Yaniv, 1971) and was kindly donated to us by Dr. A. Favre (Paris). The extent of cross-linking was determined by the fluorimetric method of Favre & Yaniv (1971) to be greater than 99%. Radioactive amino acids were purchased from Radiochemicl Centre (Amersham) and all other chemicals (analytical grade) from Merck (Darmstadt).

The amino acid acceptance of a given tRNA was determined by following the time dependence of the aminoacylation reaction (Kosakowski & Böck, 1970). The enzyme was used at nanomole concentrations. Amino acids as used were 20 μM L-[^{14}C]phenylalanine, 20 μM L-[^{14}C]isoleucine, and 50 μM L-[^{14}C]tyrosine, all having a specific radioactivity of 10 μCi/μmol. The initial rate of charging of $tRNA^{Phe}$ (yeast) was determined by the same method in the presence of 26 nM phenylalanyl-tRNA synthetase and 0.3–11 μM $tRNA^{Phe}$. The reaction buffer was 0.1 M Tris-HCl (pH 7.5 at 28 °C), 10 mM KCl, 10 mM $MgCl_2$, and 2 mM reduced glutathione. The degree of aminoacylation was measured at times between 1 and 10 min to ensure that true initial rates were determined.

The formation of a complex between phenylalanyl-tRNA synthetase and $tRNA^{Ile}$ was detected by observing the ligand-induced decrease of the intrinsic fluorescence intensity (5.5% at maximum) (Bartmann et al., 1975b). Measurements were made on a Perkin-Elmer MPF-2A fluorescence spectrophotometer thermostated at 25 ± 0.5 °C. Quartz cuvettes (4 mm × 10 mm) from Hellma (Müllheim) were used with the excitation light entering the small side. The wavelength of the excitation light was 300 nm (9-nm slit width), and the emission light was detected at a wavelength of 340 nm (10-nm slit width). The concentrations were 0.51 μM enzyme and 0.067–3.0 μM $tRNA^{Ile}$ with 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.4 mM dithioerythritol. The observed intensities were corrected for the absorbance of light by the added $tRNA^{Ile}$ by comparing the fluorescence from the sample with that from a solution in which the enzyme had been replaced by 71 μM L-tryptophan. The data analysis was based on the average of three separate titrations of both the sample and reference solutions. The reference titration curve was graphically smoothed prior to subtracting from the sample data.

Light Scattering. Samples prepared as described above were solubilized in a standard buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 0.2 mM DTT) containing substrates, tRNA, and/or salt appropriate to a given experiment. About 25 μL of each solution was loaded into a 50-μL Hamilton microsyringe fitted with a swinney adaptor to which a small volume filtering apparatus (Flath-Lundin filter, Hamilton Syringe Co.) was attached. After the first few microliters of filtrate was discarded about 10 μL, was filtered through a 0.22-μm Millipore GS membrane filter into a 2 × 2 × 40 mm quartz cuvette for light scattering at an angle of 90°. The cuvette was introduced into a temperature-controlled (20 ± 0.1 °C) light-scattering spectrometer, and data were collected as described previously (Olson et al., 1976). All buffers were prepared from particle-free water from a two-stage distillation apparatus (Gabler et al., 1975).

Protein concentrations were determined from the solutions after each measurement by using the Coomassie Brillant Blue method (Bradford, 1976). In some cases, the amount of $tRNA^{Phe}$ was determined by using the aminoacylation assay. In all cases examined, the loss of protein and tRNA during filtration through the Millipore filter was negligible.

Scattered light was detected with a photomultiplier, the single-photon pulses were amplified, and a discriminator was used to provide standardized pulses for use in a digital correlator. The output of the correlator was analyzed by using the University's computer to give the diffusion constant according to

$$g(\tau) = A + Be^{-2Dk^2\tau} \quad (1)$$

where $g(\tau)$ is the measured correlation function, A and B are the fitted parameters, D is the diffusion constant, $\kappa = [4\pi n/\lambda] \sin(\theta/2)$ is the scattering wave number (n = index of refraction, λ the wavelength of light under vacuum, and θ the scattering angle), and τ is the argument of the correlation function.

In interpreting the measured values of diffusion constants in terms of the binding constants of various ligands, we first assumed that the conformation of the enzyme was determined by the presence or absence of the ligand. That is, we assumed the interaction between ligand and enzyme followed the reaction

$$E_1 + nL \underset{K_{diss}}{\rightleftharpoons} L_n{\cdot}E_2 \quad (2)$$

[1] Abbreviations used: $tRNA^{Phe}$CCp, $tRNA^{Phe}$ lacking the terminal adenosine; $tRNA^{Phe}$CC, $tRNA^{Phe}$ lacking the terminal AMP; DTT, dithiothreitol.

where the subscripts denote different conformations. K_{diss} is the dissociation constant of the enzyme·ligand complex. In this case, we expect to observe values of the diffusion constant at zero ligand concentration characteristic of the conformation E_1 and at high ligand concentration characteristic of E_2. Of all the ligands investigated, only tRNA has a molecular weight (25 000) sufficiently large in comparison to the synthetase (267 000) to lead to the possibility that the presence of the free ligand would have an effect on the value of the measured diffusion constant. With equimolar quantities, the ratio of the intensity of light scattered by the enzyme to that scattered by the tRNA would be $(267\,000/25\,000)^2[(\partial n/\partial C)_{enzyme}/(\partial n/\partial C)_{tRNA}]^2$. The second factor is close to 1 so that the scattering-intensity ratio is about 114. Thus, we would expect to be able to use a 10-fold excess of tRNA without significantly influencing the diffusion constant measurements. By intentionally adding larger amounts of tRNA, we were able to show that more than a 30-fold excess of tRNA was required to produce an observable change in the measured diffusion constant.

We conclude that a 10-fold excess of tRNA, the largest tRNA concentration used in the experiments reported here, represents a conservative upper limit.

In order to measure the relative concentrations of the two conformations, we fit a single exponential with decay constant $2DK^2$ to the data which were assumed to be in reality made up exponentials of decay constants $2D_1K^2$, $(D_1 + D_2)K^2$, and $2D_2K^2$ (we have assigned the symbols C_1 and D_1 to the concentration and diffusion constant in conformation 1 and C_2 and D_2 for conformation 2). When an analytic least-squares fit of the single exponential to the three exponentials weighted by the concentrations $C_1^2:2C_1C_2:C_2^2$ is done, it is found (C.-C. Wang et al., unpublished results) that the measured diffusion constant representing the weighted average for enzymes in both states will be given by the solution to

$$\frac{C_1^2(D - D_1)}{(D + D_1)^2} + \frac{4C_1C_2[2D - D_1 - D_2]}{[2D + D_1 + D_2]^2} + \frac{C_2^2(D - D_2)}{(D + D_2)^2} = 0 \tag{3}$$

As long as D_1 and D_2 are not greatly different, this equation may be reduced with an accuracy adequate to our purposes to the simpler form

$$D = D_1 + \frac{C_2}{C}(D_2 - D_1) \tag{4}$$

where $C = C_1 + C_2$ is the total enzyme concentration. Equation 4 may then be used to convert measured diffusion constants into relative conformation population values, C_2/C.

We define the dissociation constant for the reaction of eq 2 as

$$K_{diss} = \frac{C_1C_L^n}{C_2} \tag{5}$$

which may be rewritten as

$$\frac{K_{diss}}{C_L^n} = \frac{C}{C_2} - 1 \tag{6}$$

Defining $D - D_1 = \delta$ and $D_2 - D_1 = \Delta$ eq 6 becomes

$$\frac{K_{diss}}{C_L^n} = \frac{\Delta}{\delta} - 1 \tag{7}$$

Thus, a log–log plot of the quantity $(\Delta/\delta) - 1$ vs. $1/C_L$ will in principle determine both the dissociation constant K_{diss} and the number n. Because of the scatter in our data, we averaged several points before determining K_{diss} and n. We believe the values of K_{diss} as determined in this way to be accurate to 50%. All of the data are consistent with the value $n = 1$ which we have used in the analysis.

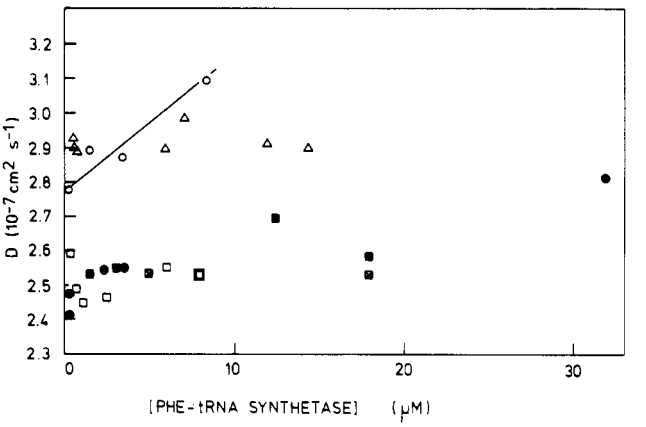

FIGURE 1: Diffusion constant as a function of the concentration of phenylalanyl-tRNA synthetase. tRNA is not present. Conditions: 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.2 mM dithiothreitol. (○) No added salt. The slope of the line was used to calculate the number of charges carried by the protein according to eq 8. Other conditions included the following: (△) 10 mM $MgCl_2$ plus $tRNA^{Phe}$ in a 5-fold excess over enzyme, (●) 166 mM NaCl, (□) 10 mM $MgCl_2$, (□) 20 mM $MgCl_2$, and (■) 10 mM $MgCl_2$ plus 3 mM L-phenylalanine. The temperature was 20 °C. No lines were drawn for these conditions.

The preparations of $tRNA^{Ile}$, $tRNA^{Tyr}$, $tRNA^{Phe}CCp$, and $tRNA^{Phe}$ CC contained traces of $tRNA^{Phe}$, which could cause a change in the measured diffusion constant that would be falsely attributed to the other tRNAs investigated. In order to rule out this possibility, the synthetase was titrated with the preparation of the tRNA of interest. The stoichiometry was determined by applying concentrations of enzyme (2–10 μM) that would be sufficiently high to bind the $tRNA^{Phe}$ in the preparations. Resulting stoichiometries were all in the range 1–2 mol of tRNA/mol of synthetase and cannot be attributed to the traces of $tRNA^{Phe}$. It is concluded that the other types of tRNA investigated bind to the synthetase and give rise to almost all of the observed change of the diffusion constant. From the resulting binding curves, an upper estimate of the dissociation constants of the particular enzyme·tRNA complexes was obtained by taking the concentrations of tRNA at half-maximal change in D.

Results

We first measured the diffusion constant of phenylalanyl-tRNA synthetase as a function of the enzyme concentration. A weak concentration dependence in D is expected for any macromolecule having a net electric charge (Olson et al., 1976; C.-C. Wang et al., unpublished results). The magnitude of the effect depends upon the electric charge of the macromolecule, Z, and the ionic strength of the solution, μ, according to the approximate relation

$$\frac{1}{D_0}\frac{\Delta D}{\Delta C} = 0.5\frac{Z^2}{\mu} \tag{8}$$

where $\Delta D/\Delta C$ is the change in diffusion constant corresponding to a change in the concentration (in mol/L) of the macromolecule. The results of the measurements made in solutions of several different ionic strengths are shown in Figure 1 where the general features predicted by eq 8 are observed. Data approximated by a line are compatible with eq 8 within experimental error with $Z = 11 \pm 3$. The net charge is negative since the isoelectric point for this molecule is known to be approximately 4.5 (T. Hanke and E. Holler,

Table I: Diffusion Constants of Various Phenylalanyl-tRNA Synthetase·Ligand Complexes Determined by Laser Light Scattering

ligand	concentration range	solution conditions[a]	D_1 (10^{-7} cm²/s)[h]	D_2 (10^{-7} cm²/s)[h]	K_{diss} (μM)
$MgCl_2$	0–100 mM		2.85	2.40	3300 ± 1000
$tRNA^{Phe}$	0.19–48 μM	10 mM $MgCl_2$	2.40	2.95	0.25 ± 0.2 0.2[e]
$tRNA^{Phe}$	1.5–15 μM	1 mM $MgCl_2$	2.78	same	
L-phenylalanine	1–3 mM	10 mM $MgCl_2$	2.55	same	
MgATP ([Mg^{2+}]:[ATP] = 1:1 mol/mol)	2 mM	8 mM $MgCl_2$	2.45	same	
MgATP plus L-phenylalanine (formation of enzyme·phenylalanyladenylate complex)	2 mM MgATP and 4.4 mM L-phenylalanine	10 mM $MgCl_2$ and 2 nM inorganic pyrophosphatase	2.45	2.66[b]	
$tRNA^{Tyr}$	15–32 μM	10 mM $MgCl_2$	2.49	same	
$tRNA^{Phe}$ (yeast)	2–7 μM	10 mM $MgCl_2$	2.45	2.75	9 ± 2; 8 ± 2[f]
$tRNA^{Phe}_{h\nu}$	1–75 μM	10 mM $MgCl_2$	2.45	2.75	5 ± 3; 6.3 ± 0.1[g]
$tRNA^{Phe}$CC (missing terminal pA)	0.2–62 μM	10 mM $MgCl_2$	2.45	2.95	<2[c]
$tRNA^{Phe}$CC (missing terminal A)	0.5–89 μM	10 mM $MgCl_2$	2.45	2.95	<6[d]
$tRNA^{Ile}$	5–31 μM	10–20 mM $MgCl_2$	2.45	2.86	

[a] All solutions contained, in addition, 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM DTT. The temperature was 20 °C. All tRNAs were from *E. coli* unless otherwise noted. [b] The diffusion constant was determined at various intervals between 1 and 15 min from the time of mixing the reactants. [c] The tRNA concentration was comparable to that of the enzyme. A stoichiometry of enzyme:$tRNA^{Phe}$CC of 1:1 was observed. [d] Same as under footnote *c*. A stoichiometry of 1:2 was observed. [e] Conditions: 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.4 mM dithioerythritol, and 11 mM $MgCl_2$ (Bartmann et al., 1975a). [f] Michaelis-Menten constant at 28 °C, 0.1 M Tris-HCl (pH 7.5). For conditions of the aminoacylation reaction see Materials and Methods. [g] Michaelis-Menten constant at 28 °C, 0.1 M Tris-HCl (pH 7.5). [h] Diffusion constants have an averaged experimental error of 0.05 × 10^{-7} cm²/s, determined from sets of 5–10 measurements.

unpublished results). The data in the remainder of this paper were taken at enzyme concentrations ranging from 0.03 to 10 μM at ionic strengths between 5 and 200 mM. In experiments dealing with the effects of tRNA and Mg^{2+}, the total changes in D as a function of enzyme concentration are small in comparison to the experimental uncertainty (Figure 1). Consequently, we have not attempted to correct the data in the remainder of this paper for enzyme concentration effects.

We have studied extensively the effect of $MgCl_2$ on the diffusion constant and, to a lesser extent, the effect of NaCl. The results, presented in Figure 2, show a marked decrease in D as the concentration of both salts is increased. However, the character of the decrease is quite different in the two cases. The decrease in D as NaCl is added to the solution shows no saturation effect even at the higher concentration investigated (165 mM). In contrast, the decrease in D as MgCl is added is nearly completed at 10 mM $MgCl_2$; further addition up to 100 mM has little effect on D.

When the analysis techniques described earlier are used, the $MgCl_2$ data may be described in terms of the reaction

$$Mg^{2+} + E_1 \underset{K_{diss}}{\rightleftharpoons} E_2(Mg^{2+}) \qquad (9)$$

The solid curve in Figure 2 gives the theoretical result for the reaction of eq 9 with the parameters $D_1 = (2.85 \pm 0.08) \times 10^{-7}$ cm²/s, $D_2 = (2.40 \pm 0.08) \times 10^{-7}$ cm²/s, and $K_{diss} = 3.3 \pm 1$ mM. It is seen that the data are fit quite well by this theory. We have made no similar attempt to analyze the NaCl data because our results suggest that Na^+ does not bind specifically but rather acts unspecifically as an electrolyte.

When $tRNA^{Phe}$ is added to the solution in the presence of 10 mM $MgCl_2$, it is found that the diffusion constant increases from 2.40 × 10^{-7} cm²/s in the absence of $tRNA^{Phe}$ to the high concentration value of 2.95 × 10^{-7} cm²/s (Figure 3). Once again the binding constant may be estimated for the reaction

$$tRNA^{Phe} + E_1 \underset{K_{diss}}{\rightleftharpoons} E_2{\cdot}tRNA^{Phe} \qquad (10)$$

The theoretical curve for the parameters $D_1 = (2.40 \pm 0.8) \times 10^{-7}$ cm²/s, $D_2 = (2.95 \pm 0.8) \times 10^{-7}$ cm²/s, and $K_{diss} = 0.25 \pm 0.2$ μM is included in Figure 3 as the solid line. When, however, tRNA is added to the enzyme in a solution containing only 1 mM $MgCl_2$, there is no clear change in D (Figure 3). The data give an average value of $D = (2.78 \pm 0.08) \times 10^{-7}$ cm²/s (five data points) which may be compared to the average value of $D = (2.93 \pm 0.06) \times 10^{-7}$ cm²/s (five data points) in 10 mM $MgCl_2$ for all concentrations of $tRNA^{Phe}$ over 1 μM.

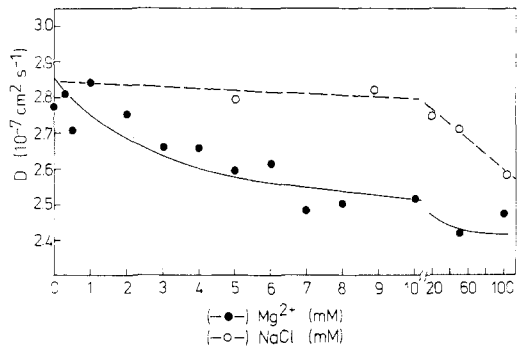

FIGURE 2: Diffusion constant of phenylalanyl-tRNA synthetase as a function of Mg^{2+} concentration. tRNA is not present. Conditions: 10 mM Tris-HCl buffer, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.2–2.0 μM synthetase. Closed circles are for Mg^{2+} ions and open circles for NaCl. The temperature was 20 °C. The line was computed on the basis of

$$D = 2.85 \times 10^{-7} - \frac{0.45 \times 10^{-7}}{1 + \frac{3.3 \times 10^{-3}}{C_{Mg^{2+}}}} \text{ (cm}^2\text{ s}^{-1}\text{)}$$

The concentration of Mg^{2+}, $C_{Mg^{2+}}$, is mol/L.

In addition to the three experiments described above, we have determined the influence of a number of ligands upon the diffusion constant of the enzyme. The results of all experiments are summarized in Table I where we present the ligand and the concentration range, the solution conditions, and the parameters D_1, D_2, and K_{diss}. In each case, D_1 is the diffusion constant in the absence of the ligand, D_2 the limiting

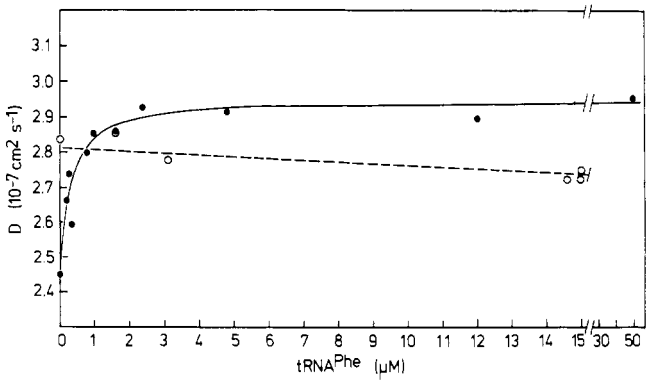

FIGURE 3: Diffusion constant of phenylalanyl-tRNA synthetase as a function of $tRNA^{Phe}$ concentration. Conditions: 10 mM Tris-HCl buffer (pH 7.5, 20 °C), 0.1 mM EDTA, and 1 mM dithioerythritol. The concentration of tRNA was at most 10-fold in excess of the synthetase. (●) 10 mM $MgCl_2$; the curve has been computed on the basis of

$$D = 2.40 \times 10^{-7} + \frac{0.55 \times 10^{-7}}{1 + \frac{0.25 \times 10^{-6}}{C_{tRNA^{Phe}}}} \ (cm^2\ s^{-1})$$

The concentration of $tRNA^{Phe}$, $C_{tRNA^{Phe}}$, is mol/L. (○) In the presence of 1 mM $MgCl_2$.

diffusion constant at high ligand concentration, and K_{diss} the dissociation constant for the reaction

$$L + E_1 \underset{K_{diss}}{\rightleftharpoons} E_2{\cdot}L \qquad (11)$$

which is given only in those cases for which a change in D is detected. While we have analyzed all results by using the value $n = 1$ in eq 2, the data are not sufficiently good to rule out the value $n = 2$ in most cases. Reactions with values of greater than 2 would not be compatible with the data. The titration of phenylalanyl-tRNA synthetase with $tRNA^{Ile}$ was followed by measurement of the fluorescence intensity at 340 nm. In Figure 4, the decrease of the intensity is plotted after correction for absorbance effects as a function of [$tRNA^{Ile}$]. Experimental points follow closely the two lines, which fit at low and high concentrations, indicating relatively tight binding of $tRNA^{Ile}$ ($K_{diss} < [E]_0 = 0.51\ \mu M$; Bartmann et al., 1975b). The point of intersection is $0.49 \pm 0.05\ \mu M$ $tRNA^{Ile}$, indicating a 1:1 stoichiometry of the enzyme·$tRNA^{Ile}$ complex.

The Michaelis–Menten constant for $tRNA^{Phe}$ (yeast) in the aminoacylation reaction catalyzed by phenylalanyl-tRNA synthetase (*E. coli*) was determined to be $8.1 \pm 0.9\ \mu M$. A plot of the initial reaction velocity vs. the ratio of the velocity over the initial concentration of tRNA (Eadie, 1942) followed a linear dependence (not shown), indicating a single class of reacting centers on the enzyme. The catalytic rate constant was 0.14 s^{-1}.

Table II: Values of the Frictional Factor Ratio and of the Effective Radius of the Macromolecule and Macromolecule Complex, Calculated from the Stokes–Einstein Equation and from Equation 12[a]

solution conditions	$M_w \times 10^{-3}$	D (10^{-7} cm² s^{-1})	f/f_0	R_{eff}
no salt, no tRNA	267	2.85	1.79	76.8 Å
10 mM Mg^{2+}; no tRNA	267	2.40	2.13	91.3 Å
10 mM Mg^{2+}; $tRNA^{Phe}$	317	2.95	1.64	74.2 Å
10 mM Mg^{2+}; ±Phe	267	2.40	2.13	91.3 Å
10 mM Mg^{2+}; $tRNA^{Phe}$ (yeast)	317	2.75	1.76	79.6 Å

[a] The M_w of the tRNA was assumed to be approximately 25 000. The molar ratio of tRNA to enzyme was assumed to be 2:1. The M_w given in the table refers to that of the enzyme·substrate complex.

Discussion

Diffusion Constant and Molecular Shape. The measurement of the diffusion constant allows the following statements about molecular weight and molecular shape of the enzyme.

(1) Using our value of D in zero added salt, the value of the sedimentation coefficient $S = 8.6$ (Hanke et al., 1974), the value of the partial specific volume of the protein $\bar{v} = 0.74$ cm^3/g (Hanke et al., 1974), and the value for the density of water, we calculate the molecular weight for the synthetase according to the Svedberg equation (Tanford, 1961), $M_w = 291\,000$. This value is in agreement with the value determined by Hanke et al. (1974) of 267 000.

(2) The most convenient measure of the protein shape is the ratio

$$\frac{D_{sphere}}{D} = \frac{R_{eff}}{R_0} = \frac{f}{f_0} \qquad (12)$$

where D_{sphere} and R_0 are the diffusion constant and radius of a hard sphere with the same density and molecular weight as the enzyme; D and R_{eff} refer to the real molecule with D the measured diffusion constant and R_{eff} the corresponding radius. R_{eff} can be calculated from the Stokes–Einstein equation (Ford, 1972). f/f_0 is the friction factor ratio familiar from sedimentation studies (Tanford, 1961). R_0 is calculated with respect to two tRNA molecules per one molecule of enzyme (Bartmann et al., 1975) when M_w of the complex is considered. The results for f/f_0 and for R_{eff} are summarized in Table II.

(3) Each value of f/f_0 may be used to determine the axial ratio of an equivalent ellipsoid of revolution (Tanford, 1961)

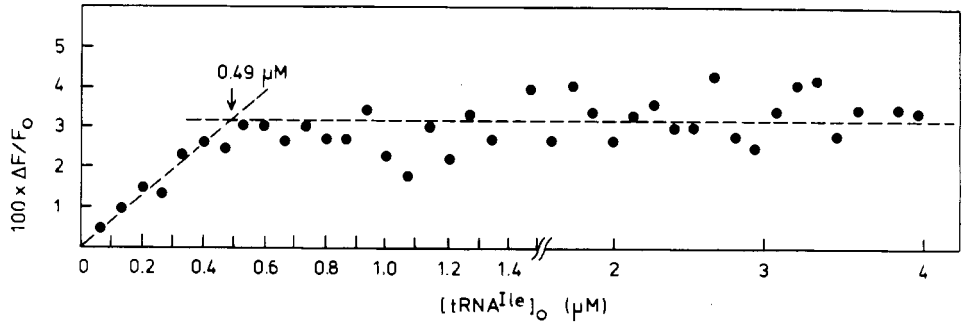

FIGURE 4: Formation of a complex between phenylalanyl-tRNA synthetase and $tRNA^{Ile}$ as followed by its effect on the intensity of the protein-intrinsic fluorescence. Because tRNA absorbs light in the wavelength range applied, the measured fluorescence intensity at a given concentration of tRNA was compared with that of a solution of tryptophan under identical conditions and the enzyme absent. The concentration of tryptophan was such that initial fluorescence intensities (tRNA absent) were identical for tryptophan and the enzyme. The value of ΔF obtained from the comparison was divided by the actual intensity of the Trp fluorescence (at the concentration of tRNA), F_0. The ratio of $\Delta F/F_0$ is plotted as a function of total concentration of $tRNA^{Ile}$.

providing the extent to which the molecule is hydrated is known. Values of the axial ratio near 1 provide good evidence that the molecule is globular in shape while values in excess of 10 are indicative of extended molecules. If we assume there is no water of hydration, the axial ratio is 15 with no Mg^{2+} and 24 with 10 mM Mg^{2+} but no tRNA. If the volume of the hydration shell is 50% of the volume of the molecule, these ratios are 10 and 17. Since estimated hydration volumes for proteins rarely exceed 40%, we see that the molecule is elongated under all solution conditions and that the addition of Mg^{2+} results in an even more asymmetric form.

Cases of unusually high f/f_0 values have been reported for other proteins capable of binding nucleic acids. Examples are aminoacyl-tRNA synthetases specific for glycine (Surguchov & Surguchova, 1975) and for L-leucine of yeast (Chirikjian et al., 1973), DNA polymerase of rat liver (Holmes & Johnston, 1973) and of *Physarum polycephalum* (Baer & Schiebel, 1978), and the T4 bacteriophage gene-32 protein (Delius et al., 1972).

Effect of NaCl and $MgCl_2$. NaCl and $MgCl_2$ have qualitatively similar effects on the diffusion constant of phenylalanyl-tRNA synthetase. Both salts result in a reduced value of D. This behavior is atypical for the effect of salt on the hydrodynamic size of a macroion—it is more usual to find the molecule at its largest size in low-salt solutions because the mutual replusion of various parts of the molecule is stronger in the absence of salt (Tanford, 1961). The present results show that in this enzyme the dominant interaction maintaining the tertiary structure takes place between regions of the molecule having opposite charge. The presence of small ions reduces this interaction, allowing the molecule to expand.

It is clear from the plots of D vs. salt concentration that while the NaCl probably acts only as an electrolyte, the Mg^{2+} is bound to the enzyme in such a way as to hold it in the more open state. An ionic strength of more than 160 mM in NaCl is required to achieve the same change in D as results from an ionic strength of only 30 mM in $MgCl_2$ (a concentration of 10 mM $MgCl_2$). This result provides the final direct evidence of the influence of Mg^{2+} on the conformation of phenylalanyl-tRNA synthetase; circular dichroism and intrinsic fluorescence experiments (Pimmer & Holler, 1979) show no direct effect. There are, however, several experiments that provide indirect evidence of a Mg^{2+}-induced conformational change. (1) An active site directed fluorescent indicator, 2-*p*-toluidinylnaphthalene-6-sulfonate, exhibits an increased intensity of light emission upon titration of the enzyme–indicator complex with Mg^{2+} (Hanke et al., 1975; Pimmer & Holler, 1979). The titration curve is biphasic. The K_{diss} = 4.1 ± 0.5 mM of the low affinity binding site (Pimmer & Holler, 1979) agrees with K_{diss} = 3.3 ± 1 mM obtained from analysis of the diffusion constants (Figure 2). (2) The amino acid activation (the synthesis of the phenylalanyladenylate) is stimulated by Mg^{2+} in the millimolar concentration range (Bartmann et al., 1975a; Pimmer & Holler, 1979). (3) The phenylalanyl-tRNA synthetase stimulated conformational change of $tRNA^{Phe}$ depends on millimolar concentrations of Mg^{2+} (Favre et al., 1979). (4) Mg^{2+} ions offer protection against modification of phenylalanyl-tRNA synthetase by sulfhydryl- and histidine-specific reagents (Kosakowski & Böck, 1971; Hennecke & Böck, 1974). (5) Mg^{2+} ions affect the rate of reactivation of phenylalanyl-tRNA synthetase after exposure to denaturation at pH 2 (Hanke et al., 1975).

The results in Table I show that Mg^{2+} ions at 10 mM are required for tRNA to have an effect on D. Stimulation by Mg^{2+} ions in the same concentration range has been observed for a variety of reactions, including the protection of $tRNA^{Phe}$ by phenylalanyl-tRNA synthetase against UV-light-induced cross-linking (Favre et al., 1979) and for acceleration of phenylalanylation of $tRNA^{Phe}$ (Pimmer & Holler, 1979). From all of these results, it appears unlikely that the conformation of free enzyme (in the absence of Mg^{2+} ions) and the enzyme·Mg^{2+}·$tRNA^{Phe}$ complex are the same, although the values of D are similar (Table I). Despite the observation that the effects of Mg^{2+} and $tRNA^{Phe}$ on D are similar in magnitude but opposite in sign, a mechanism which relates this effect to tRNA pulling the Mg^{2+} ions from the enzyme seems to be ruled out because Mg^{2+} ions have been shown to bind to the synthetase in the presence of $tRNA^{Phe}$ via the same dissociation constants as in the absence of $tRNA^{Phe}$ (Pimmer & Holler, 1979).

Effect of tRNA. The addition of tRNA to a solution containing enzyme and Mg^{2+} causes the value of D for the enzyme to increase to the value for the enzyme in the absence of either Mg^{2+} or tRNA. It is tempting to interpret the equal but opposite effects of Mg^{2+} and tRNA in terms of an expansion and contraction of the enzyme. A similar explanation has been given for the results of neutron scattering in the methionine-specific system of *E. coli* (Dessen et al., 1978). A contraction upon binding tRNA has been reported for the yeast Phe enzyme (Pilz et al., 1979).

The values of the dissociation constants of the enzyme·tRNA complexes as measured by the changes in the diffusion constant (Table I) can be used in comparison with values from other techniques to derive the following conclusions. (1) The values measured here are compatible with the values of the Michaelis–Menten constants for the aminoacylation catalyzed under similar conditions (Table I, the Michaelis–Menten constant for $tRNA^{Phe}$ is 0.26 μM; Stulberg, 1967). For $tRNA^{Phe}$, an agreement is seen with the value of the dissociation constant, which has been determined via the enzyme intrinsic fluorescence. (2) The observed changes in D reflect binding of tRNA to single types of sites as has been reported in the case of changes in the enzyme intrinsic fluorescence but that is in contrast to gel equilibrium filtration at 1 mM Mg^{2+}, which indicates an anticooperative binding of two molecules of $tRNA^{Phe}$ to each molecule of enzyme (Bartmann et al., 1975a,b). Whether these observations reflect an asymmetry in the two binding sites of the enzyme·$(tRNA^{Phe})_2$ complex has to be established. (3) The effect on D does not require the 3′-terminal pA of the tRNA. Further studies would be of interest with respect to the minimum size of a tRNA fragment able to induce the conformational change. (4) The observed effect is not restricted to cognate tRNA as is seen for $tRNA^{Ile}$. The enzyme intrinsic fluorescence is another property sensitive to this interaction (Figure 4). Interestingly, $tRNA^{Ile}$ has been reported to be phenylalanylated in the system of yeast (von der Haar & Cramer, 1978). (5) The extent to which the diffusion constant is changed seems to correlate with the affinity of the enzyme and tRNA to form a complex, if the Michaelis–Menten constants for $tRNA^{Phe}$ (yeast) and $tRNA^{Phe}_{h\nu}$ are assumed to represent values close to those of the dissociation constants (Table I). A tRNA which does not interact such as $tRNA^{Tyr}$ has no effect on D.

Recently, a tRNA-induced conformational change has been postulated on the basis of rapid kinetics and has been suggested to control, at least in part, the recognition between a synthetase and a tRNA (Krauss et al., 1976, 1979). In contrast with these results, the conformational change observed by the light scattering is within experimental error the same for $tRNA^{Phe}$, $tRNA^{Phe}CCp$, $tRNA^{Phe}CC$, and $tRNA^{Ile}$ and similar to that

for $tRNA_{h\nu}^{Phe}$ and $tRNA^{Phe}$ from yeast (Table I). The poor sensitivity of the diffusion constant in detecting tRNA structure variation as opposed to the changes seen with the rapid kinetic methods suggests that different conformational changes are seen with the two methods, those from kinetics being perhaps more subtle than those from light scattering. If indeed there are two separate conformational changes, whether they occur successively or in parallel remains to be established.

Of the small ligands L-phenylalanine, MgATP, and phenylalanyladenylate (formed in situ in the presence of the substrates and Mg^{2+}), only the enzymatic intermediate causes a change in *D*. The lack of a change in *D* in the other cases is certainly not at variance with the concept of rearrangements involved in substrate binding and functioning of the enzyme. These conformational changes are perhaps subtle and/or involve smaller parts of the protein. Clearly, the conformational states involved in homotropic negative cooperativity of binding of $tRNA^{Phe}$ and L-phenylalanine (Bartmann et al., 1975a) cannot be the same.

References

Baer, A., & Schiebel, W. (1978) *Eur. J. Biochem. 86*, 77–84.

Bartmann, P., Hanke, T., & Holler, E. (1975a) *Biochemistry 14*, 4777–4786.

Bartmann, P., Hanke, T., & Holler, E. (1975b) *J. Biol. Chem. 250*, 7668–7674.

Chirikjian, J. G., Kanagalingam, K., Lau, E., & Fresco, J. R. (1973) *J. Biol. Chem. 248*, 1074–1079.

Delius, H., Mantell, N. J., & Alberts, B. (1972) *J. Mol. Biol. 67*, 341–350.

Dessen, P., Blanquet, S., Zaccai, G., & Jacrot, B. (1978) *J. Mol. Biol. 126*, 293–313.

Eadie, G. S. (1942) *J. Biol. Chem. 146*, 85–93.

Favre, A., & Yaniv, M. (1971) *FEBS Lett. 17*, 236–240.

Favre, A., Ballini, J. P., & Holler, E. (1979) *Biochemistry 18*, 2887–2895.

Ford, N. C. (1972) *Chem. Scr. 2*, 193–206.

Gabler, R., Ford, N. C., & Westhead, E. W. (1975) *Biophys. J. 15*, 747–751.

Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., & Tener, G. H. (1967) *Biochemistry 6*, 3043–3056.

Hanke, T., Bartmann, P., Hennecke, H., Kosakowaski, H. M., Jaenicke, R., Holler, E., & Böck, A. (1974) *Eur. J. Biochem. 43*, 601–607.

Hanke, T., Bartmann, P., & Holler, E. (1975) *Eur. J. Biochem. 56*, 605–615.

Hennecke, H., & Böck, A. (1974) *Eur. J. Biochem. 50*, 157–166.

Holler, E. (1976) *J. Biol. Chem. 251*, 7717–7719.

Holmes, A. M., & Johnston, I. R. (1973) *FEBS Lett. 29*, 1–6.

Khym, J. X., & Uziel, M. (1968) *Biochemistry 7*, 422–426.

Kosakowski, M. H., & Böck, A. (1970) *Eur. J. Biochem. 12*, 67–73.

Kosakowski, M. H., & Böck, A. (1971) *Eur. J. Biochem. 24*, 190–200.

Krauss, G., Riesner, D., & Maass, G. (1976) *Eur. J. Biochem. 68*, 81–93.

Krauss, G., von der Haar, F., & Maass, G. (1979) *Biochemistry 18*, 4755–4761.

Ohta, T., Shimada, I., & Imahori, K. (1967) *J. Mol. Biol. 26*, 519–524.

Olson, T., Fournier, M. J., Langley, K. H., & Ford, N. C. (1976) *J. Mol. Biol. 102*, 193–203.

Pearson, R. L., Weiss, J. F., & Kelmers, A. D. (1971) *Biochim. Biophys. Acta 228*, 770–774.

Pilz, I., Goral, K., & von der Haar, F. (1979) *Z. Naturforsch., C: Biosci. 34C*, 20–26.

Pimmer, J., & Holler, E. (1979) *Biochemistry 18*, 3714–3723.

Riesner, D., Pingoud, A., Boehme, D., & Maass, G. (1976) *Eur. J. Biochem. 68*, 71–80.

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

Stulberg, M. P. (1967) *J. Biol. Chem. 242*, 1060–1064.

Surguchov, A. P., & Surgochova, I. G. (1975) *Eur. J. Biochem. 54*, 175–184.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.

von der Haar, F., & Cramer, F. (1978) *Biochemistry 17*, 4509–4514.

Willick, G. E., & Kay, C. M. (1976) *Biochemistry 15*, 4347–4352.

Zaccai, G., Morin, P., Jacrot, B., Moras, D., Thierry, & Giegé, R. (1979) *J. Mol. Biol. 129*, 483–500.

Zubay, G. (1962) *J. Mol. Biol. 4*, 347–356.