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Tyrosyl-tRNA Synthetase from *Escherichia coli.* Stoichiometry of Ligand Binding and Half-of-the-Sites Reactivity in Aminoacylation†

Ross Jakes and Alan R. Fersht*

ABSTRACT: The tyrosyl-tRNA synthetase from *Escherichia coli* binds only 1 mol of tRNA, tyrosine, and tyrosyl adenylate per mol of enzyme dimer. However, like the enzyme from *Bacillus stearothermophilus,* once one active site is occupied by tyrosyl adenylate the other becomes accessible to bind a further molecule each of tyrosine and ATP. Both bacterial enzymes show biphasic kinetics with respect to tyrosine in the aminoacylation of tRNA. Equilibrium dialysis experiments show that this is due to 2 mol of tyrosine binding in the presence of ATP and tRNA. A method is given for a correction for the effects of hydrolysis of the charged tRNA on the aminoacylation kinetics.

The derivation of general principles of enzymic catalysis is greatly assisted by structure–reactivity studies. In the case of enzymes of broad steric specificity, such as chymotrypsin, the study of the nature of the protein–ligand interactions and the individual chemical steps is simplified by the ease of preparing substrates of varying structure. On the other hand, this is not possible for enzymes of high specificity such as the aminoacyl-tRNA synthetases. We are therefore undertaking a comparative study of several of these enzymes in order to derive similar information.

We have shown so far (Fersht et al., 1975a,b; Fersht, 1975) that there are similarities between the valyl activating enzyme which is a typical member of the monomeric class of enzymes of molecular weight 110,000 and the tyrosyl enzyme from *Bacillus stearothermophilus* which is a symmetrical dimer of mol wt 2 × 47,500 (Koch, 1974; Reid et al., 1973). Both enzymes exhibit negative cooperativity of ligand binding and half-of-the-sites reactivity in binding only one amino acid and apparently only one aminoacyl adenylate although possessing two sets of binding sites. The phenomena of half-of-the-sites reactivity and negative cooperativity are now well documented (Seydoux et al., 1974) but their catalytic importance is as yet unknown. We have suggested a possible catalytic mechanism, involving three limiting cases, which could explain this (Fersht, 1975).

In this context we extend the studies on the partial reactions of the tyrosyl-tRNA synthetase to cover the complete reaction of aminoacylation of tRNA. Most of the measurements concern the enzyme from *Escherichia coli* which is also a dimer of mol wt 95,000. We first show that it is mechanistically very similar to that from *B. stearothermophilus,* and then attempt to resolve the controversy between Chousterman and Chapeville (1973b) and Krajewska-Grynkiewicz et al. (1973) concerning the stoichiometry of ligand binding. Before tackling the rapid reaction studies described in the following paper (Fersht and Jakes, 1975) it is necessary to establish unambiguously the relevant stoichiometries and the relevant dissociation constants.

Experimental Section

Materials and apparatus are described by Fersht et al. (1975a) and Fersht (1975). The tyrosyl-tRNA synthetase from *E. coli* (EM 20031) was purified in the presence of phenylmethanesulfonyl fluoride (to prevent proteolysis). A detailed description will be given elsewhere (Atkinson et al., 1975). The resultant highly purified material was homogeneous by the accepted criteria showing a single band of mol

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wt 45,000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein was found to give an absorbance at 280 nm of 0.89 per mg per ml per cm, the material having been assayed by amino acid analysis after acid hydrolysis. The enzyme was stored at −20°, in 50% glycerol–water containing pH 7.5 Tris-Cl buffer, 10 m*M* mercaptoethanol, and 0.1 m*M* phenylmethanesulfonyl fluoride. tRNA from *E. coli* (obtained from M.R.E. Porton Down, Wiltshire) was enriched by chromatography on B.D.-cellulose and could be charged to 384 pmol/A_{260} (pH 7). Some material which had been further enriched by chromatography on DEAE-Sephadex was purified with the RPC-5 system (Kelmers and Heatherly, 1971) to 1000 pmol/A_{260}. [^{14}C]Tyr-tRNA was prepared by a modification of the method of Yarus and Berg (1967). A solution of $tRNA^{Tyr}$ (130 μM) was incubated with 6 m*M* ATP, 200 μM [^{14}C]Tyr (40 Ci/mol), 1 unit/ml of inorganic pyrophosphatase, and 0.7 μM tyrosyl-tRNA synthetase. After 10 min the pH of the mixture was lowered to 5 and, after phenol extraction, desalted on a G-25 column equilibrated with 5 m*M* sodium acetate (pH 5.0) and 10 m*M* $MgCl_2$.

Methods and Kinetic Procedures. Unless otherwise stated all kinetic and binding studies were performed at 25 ± 0.1° in Tris-Cl buffers (100 m*M* Tris-HCl, 44 m*M* Tris; pH 7.78) containing 10 m*M* $MgCl_2$, 10 m*M* mercaptoethanol, and 0.1 m*M* phenylmethanesulfonyl fluoride, with the tyrosyl-tRNA synthetase from *E. coli*.

Active Site Titrations. (a) The initial "burst" of ATP depletion on the addition of enzyme (2–5 μM) to a solution of [γ-^{32}P]ATP (10–20 μM) and tyrosine in the presence of inorganic pyrophosphatase was determined by the method of Fersht et al. (1975a).

(b) The following modification of the nitrocellulose filter procedure (Yarus and Berg, 1970) was used for rapid routine analysis. The enzyme (5 μl of 10–20 μM) was added to a solution (55 μl) containing 10 μM [^{14}C]tyrosine (480 Ci/mol), 1 m*M* ATP, and 1 unit of inorganic pyrophosphatase in the standard buffer. The mixture was left for 1–2 min at room temperature; aliquots (20 μl) were spotted onto the presoaked nitrocellulose filters (Schleicher and Schüll BA 85) and washed with 3.0 ml of standard buffer and dried. The amount of complex retained by the filter was assayed using a toluene based scintillant (Fersht et al., 1975a).

Preparation and Hydrolysis of Tyrosyl-tRNA Synthetase Tyrosyl Adenylate. The complex was prepared by mixing enzyme with [^{14}C]Tyr, ATP, and inorganic pyrophosphatase either under the standard conditions at pH 5.85 (substituting 100 m*M* bis-tris-Cl for the Tris-Cl) and isolated by gel filtration as described by Fersht (1975). Aliquots of the fraction containing the enzyme were dried on nitrocellulose filters and the radioactivity was measured. Similar aliquots were filtered through presoaked filters, washed with 3.0 ml of buffer, dried, and counted to determine the efficiency of retention. The rate of hydrolysis of the complex was determined from the amount retained on a nitrocellulose filter (Fersht, 1975).

Binding of Tyrosine Determined by Equilibrium Dialysis. Ten cells each of two chambers separated by a membrane (Sartorius SM 11539) constructed to the design of Englund et al. (1969) were mounted in a thermostated housing at 25° as previously described (Fersht, 1975). For the binding of tyrosine in the absence of other ligands one chamber of each cell (22 μl) was filled with 18 μM enzyme in the standard buffer. The other ten chambers contained from 10 to 250 μM [^{14}C]Tyr (59 Ci/mol). Experiments were also performed in the presence of tRNA (chargeable to 1000 pmol/A_{260}). Eight chambers contained 16 μM enzyme and 32 μM $tRNA^{Tyr}$ and the corresponding partners also 32 μM $tRNA^{Tyr}$ and from 10 to 250 μM [^{14}C]Tyr as above. The remaining two cells, containing 16 μM enzyme in the absence of tRNA, were equilibrated with either 200 or 250 μM tyrosine as controls. Similar controls were used for the determination of the binding of tyrosine in the presence of 5 m*M* ATP and inorganic pyrophosphatase (Fersht, 1975). After 2–3 hr equilibration triplicate samples of 5 μl were assayed.

Binding in Presence of tRNA under Catalytic Conditions. Larger equilibrium dialysis cells (ten) were constructed so that each chamber could hold 75 μl. Eight sets of cells contained 20 μM $tRNA^{Tyr}$, 5 m*M* pyrophosphate, 5 m*M* ATP, 6 m*M* $MgCl_2$ in pH 7.78 Tris-Cl, 10 m*M* mercaptoethanol, and 0.1 m*M* phenylmethanesulfonyl fluoride on both sides of the membrane. Both chambers in each cell also contained [^{14}C]Tyr (40–250 μM), but one chamber had 13 μM enzyme, the other 0.1 μM. After 2-hr equilibration triplicate aliquots of 10 μl were added to scintillation fluid and assayed directly. A second set of triplicate 10-μl aliquots was assayed for charged tRNA by precipitation onto nitrocellulose filters with 5% trichloroacetic acid. After washing and drying, the filters were suspended in a toluene based scintillant and assayed. The remaining two cells contained just enzyme and tyrosine for controls as above. The extent of ATP hydrolysis under the reaction conditions was determined using [^{14}C]ATP and separating the [^{14}C]AMP by chromatography on PEI sheets (Polygram) (Randerath and Randerath, 1969). The experiments were repeated using the tyrosyl-tRNA synthetase from *B. stearothermophilus* (7.5 μM) but at 5 m*M* ATP and 6–120 μM [^{14}C]Tyr.

Binding of ATP Determined from Equilibrium Gel Filtration. The method of Hummel and Dreyer (1962) was applied using a 1-ml tuberculin syringe packed with G-25 (fine) Sephadex as described by Fersht (1975) except that individual drops of eluent were collected in siliconized glass tubes. The volume of each drop (~43 μl) was measured using a 50-μl Hamilton syringe (with the tip cut off square); 35 μl of each drop was then assayed. Experiments were performed equilibrating the column with (a) 55 μM [^{14}C]ATP in the standard buffer and adding 95 μl of 18 μM enzyme in the same mixture; (b) as (a) but including 100 μM tyrosine and 1 unit/ml of inorganic pyrophosphatase in the buffer; (c) 500 μM tyrosine, 10 m*M* pyrophosphate, and 28 μM [^{14}C]ATP in the standard buffer and adding 95 μl of 15 μM $tRNA^{Tyr}$ in the same mixture.

Binding of tRNA. (a) Fluorescence Titration. 0.3 × 0.3 cm or 1.0 × 0.4 cells were used in the thermostated cell holder of Perkin-Elmer-Hitachi MPF 3 spectrofluorimeter. The protein (0.05–1.0 μM) was excited at 290 nm (2-nm band pass) and the emission at 335 nm (10-nm band pass) monitored on the progressive addition of $tRNA^{Tyr}$ (0–5 or 7 μM). Control experiments were performed substituting 25 μM tryptophan for the enzyme.

(b) Equilibrium Gel Filtration. A column of Bio-Gel P-200 (100–200 mesh, 0.8 × 18.5 cm) was equilibrated with 8–11 μM $tRNA^{Tyr}$ in the standard buffer. Enzyme (8–21 μM, 200 μl) was applied in the same mixture, 0.23- or 0.33-ml fractions were collected, and 50-μl aliquots were assayed for total $tRNA^{Tyr}$ content. The procedure was also applied to the tyrosyl-tRNA synthetase from *B. stearothermophilus* as above and also by equilibrating the column

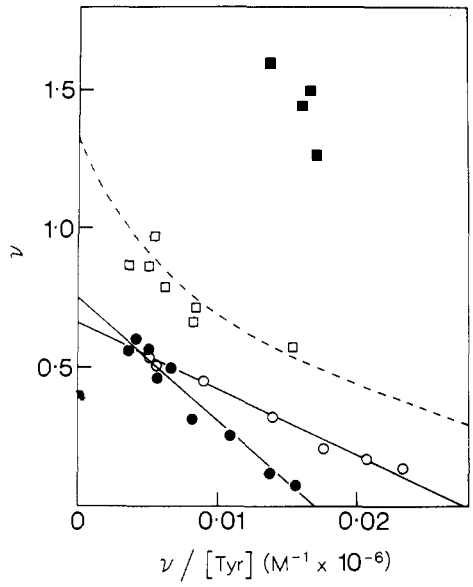

FIGURE 1: Stoichiometry (ν) of tyrosine binding to tyrosyl-tRNA synthetase from *E. coli*. (○) Tyrosine alone; (●) tyrosine in presence of tRNA; (□) in presence of $tRNA^{Tyr}$, ATP, and pyrophosphate (- - - calculated for two sites of 15 μM and 200 μM dissociation constant); (■) in presence of ATP and pyrophosphatase.

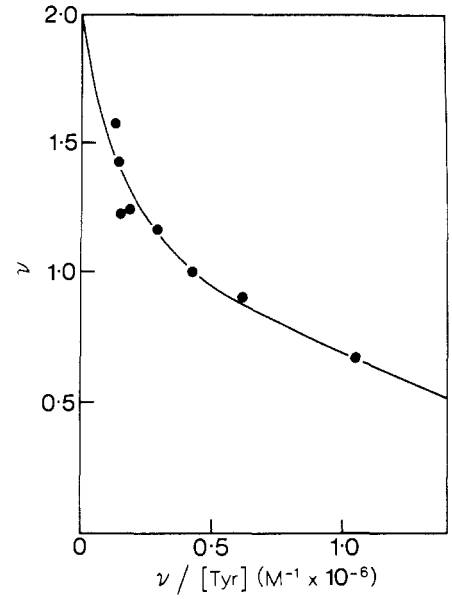

FIGURE 2: Stoichiometry of tyrosine binding to the tyrosyl-tRNA synthetase from *B. stearothermophilus* in the presence of $tRNA^{Tyr}$ (*E. coli*), ATP, and pyrophosphatase. Curve calculated for two sites of 3.6 μM and 120 μM dissociation constant.

with 250 μM tyrosine, 2.5 mM ATP, 2.5 mM pyrophosphate, and 0.2 mM AMP as well as 12 μM $tRNA^{Tyr}$ in the standard buffer. In the latter case the total $tRNA^{Tyr}$ content of each fraction could not be directly assayed since (a) some $tRNA^{Tyr}$ had become charged with cold tyrosine and (b) pyrophosphate was present; 50-μl aliquots of each fraction were therefore first equilibrated with [^{14}C]Tyr by adding 50 μl of a mixture of 10 μM [^{14}C]Tyr (specific activity 483 Ci/mol) and 20 μM tyrosyl-tRNA synthetase and incubating for 10 min (the label rapidly scrambles under these conditions as the tRNA is continuously charged and discharged); 1 unit of inorganic pyrophosphatase was then added and the charged tRNA precipitated with 5% trichloroacetic acid after 5 min.

The recovery of enzyme after gel filtration was checked and found to be greater than 95%.

Kinetics of tRNA Acylation. The K_M for tyrosine in the acylation of tRNA was determined by quenching 50-μl aliquots of a reaction mixture containing 2 mM ATP, 50 μM $tRNA^{Tyr}$, 5 units/ml of inorganic pyrophosphatase, varying concentrations of [^{14}C]Tyr, and either 1.6 nM tyrosyl-tRNA synthetase from *E. coli* or 2.4 nM ligase from *B. stearothermophilus*. The K_M for tRNA was determined as above but varying the tRNA concentration (0.8–50 μM) in the presence of 260 μM tyrosine (*B. stearothermophilus* enzyme). The deacylation rate of [^{14}C]Tyr-tRNA was also determined under the above conditions but in the absence of enzyme or alternatively in the absence of tyrosine.

Results

The enzyme lost no appreciable activity on storage in the standard buffer for a week at 4° or for 3 or 4 hr at room temperature. Concentrations were calculated using a mol wt of 95,000 and A_{280} = 0.89 per mg per ml per cm.

Stoichiometry of Tyrosyl Adenylate Binding. Active site titration using the method of Fersht et al. (1975), i.e., measuring the number of moles of ATP required to form the steady-state concentration of enzyme bound tyrosyl adenylate, gives a stoichiometry of 1.00 ± 0.05. Isolating the enzyme bound tyrosyl adenylate by gel filtration gives a value of 0.93 ± 0.05. (Experiments isolating the complex at pH 5.85 give a value of 1.07.) Nitrocellulose filtration of the complex formed in situ gives a stoichiometry of 0.82 ± 0.03. However, as the complex isolated by gel filtration was found to be only 80% retained under the conditions of the nitrocellulose filtration assay, this value may be corrected to give 1.02 ± 0.04.

Stoichiometry of Tyrosine Binding (from equilibrium dialysis, see Figure 1). In the absence of other ligands tyrosine binds to the enzyme with a dissociation constant of 24 μM and a stoichiometry of 0.66 for the concentration range 3–103 μM. The addition of 2 equiv of highly purified $tRNA^{Tyr}$ to the enzyme increases the dissociation constant to 45 μM with a stoichiometry of 0.72. In the presence of 10 mM ATP and inorganic pyrophosphatase up to 1.6 mol of tyrosine bind.

Binding under Catalytic Conditions. The binding of tyrosine to the enzyme in the presence of ATP and tRNA was measured by equilibrium dialysis by adding pyrophosphate to reverse the acylation reaction by mass action. Both chambers in each set of cells contained the 4 mM ATP, 5 mM pyrophosphate, and 20 μM tRNA and also from 37 to 230 μM (free) [^{14}C]Tyr. One chamber contained 13 and the other 0.1 μM enzyme. The chambers were balanced in this way to minimize artefacts by having identical tRNA concentrations on both sides and also enzyme to ensure acylation. At the end of the 2-hr equilibration the chamber containing 13 μM enzyme had from 3 to 5 μM acylated tRNA and the other 1.4–4.4 μM. Under these conditions chromatography of the ATP showed that less than 15% had been converted to AMP. It is seen in Figure 1 that the stoichiometry of tyrosine binding is higher than in the absence of ATP and pyrophosphate. The values may be fitted to a curve involving two sites with apparent dissociation constants 15 and 200 μM (as later found from the kinetics of tRNA acylation). These data by themselves are not convincing. Repetition of the experiments using the enzyme from *B. stearothermophilus* gave much clearer results (Fig-

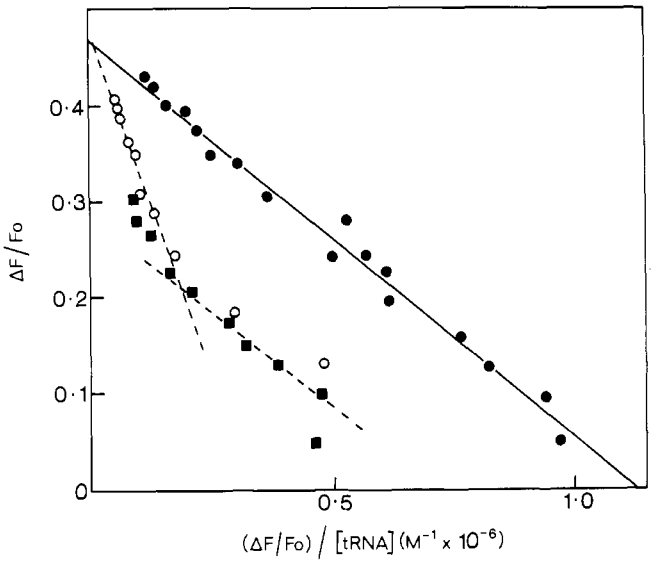

FIGURE 3: Fluorescence quenching on addition of $tRNA^{Tyr}$ to tyrosyl-tRNA synthetase from *E. coli*. (●) 0.1 μM enzyme, 0.05–3.7 μM tRNA; (■ and ○) 0.52 μM enzyme, 0.1–7.5 μM tRNA. The plot is calculated for the unbound tRNA.

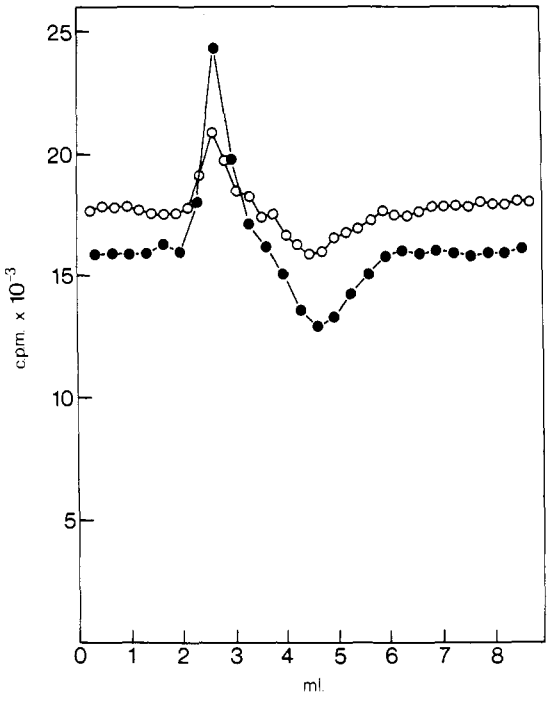

FIGURE 4: Determination of binding of $tRNA^{Tyr}$ to tyrosyl-tRNA synthetase from *E. coli* by equilibrium gel filtration. (●) 200 μl of 21 μM enzyme added to buffer containing 9 μM $tRNA^{Tyr}$; (○) 200 μl of 8 μM enzyme added to 9.9 μM $tRNA^{Tyr}$.

ure 2). The Scatchard plot is clearly curved giving a stoichiometry of greater than 1; the highest observed value being 1.6. The data fit a two-site model with dissociation constants 3.6 and 120 μM.

Binding of ATP (equilibrium gel filtration). No binding of ATP was observed in the absence of added ligands using 55 μM ATP and 18 μM enzyme. The dissociation constant of the complex must be greater than about 400 μM. Also no binding was observed under "catalytic" conditions (500 μM tyrosine, 10 mM pyrophosphate, 15 μM $tRNA^{Tyr}$, and 15 μM enzyme). Again the dissociation constant must be high (>200 μM). In the presence of inorganic pyrophosphatase and 110 μM tyrosine 1.2 ± 0.2 mol of ATP (or AMP) bind to the enzyme.

Binding of tRNA. The fluorescence emission (F) of the protein on the addition of tRNA was corrected for the absorbance of the tRNA at 290 and 335 nm by using the formula $\log F_{corr} = \log F + 0.5lA_{290} + 0.5l'A_{335}$ where l is the path length of the cell along the direction of the excitation beam and l' that for the emission (cf. Ehrenberg et al. (1971) case b). Corrections were also made for dilution. In a control experiment the apparent quenching of the fluorescence of tryptophan on the addition of tRNA was exactly accounted for by this procedure. At low concentrations of enzyme (0.05–0.1 μM) there is a 46% decrease in fluorescence on the addition of $tRNA^{Tyr}$ fitting a single dissociation constant of about 0.3–0.4 μM (see Figure 3). However, at higher enzyme concentrations (0.5–1 μM) a biphasic plot of $(\Delta F/F_0)$ vs. $(\Delta F/F_0)/[tRNA]$ is observed extrapolating again to about 46% quenching, but with a second higher dissociation constant of 1.5–2 μM. Odd concentration effects have been observed in a careful fluorescence study by Rigler et al. (1971). Also Blanquet et al. (1973) found some inexplicable results. We therefore turned to equilibrium gel filtration to resolve the question here. Three experiments, equilibrating the column with 9–10 μM $tRNA^{Tyr}$, gave values of 0.66, 0.79, and 0.68 for the stoichiometry of tRNA binding (Figure 4). A value of 0.87 was found for the binding of the tRNA (19 μM) to the enzyme from *B. stearothermophilus*. Under catalytic conditions (250 μM tyrosine, 2.5 mM ATP, 0.2 mM AMP, 2.5 mM pyrophosphate, and 12.3 μM tRNA), 0.81 mol of tRNA bound/mol of enzyme (*B. stearothermophilus*).

Kinetics of Acylation of tRNA. There is an appreciable rate of hydrolysis of Tyr-tRNA under the reaction conditions ($t_{1/2}$ = 21 min)

$$\text{tRNA} \xrightarrow{V} \text{Tyr-tRNA} \xrightarrow{k_h} \text{Tyr} + \text{tRNA}$$

If the rate of formation of aminoacyl-tRNA is V and the subsequent hydrolysis rate is k_h [Tyr-tRNA] then it may be shown that, if $[\text{Tyr-tRNA}]_t$ is the amount of acylated tRNA at time t,

$$V = [\text{Tyr-tRNA}]_t k_h (1 - e^{-k_h t})^{-1}$$

The value of k_h under our reaction conditions is 5.52×10^{-4} sec^{-1}. A plot of V against $V/[\text{Tyr}]$ for the acylation of tRNA by the tyrosyl-tRNA synthetase from *E. coli* (Figure 5) shows two K_M values for tyrosine, and two values of k_{cat}, 15 μM and 6.33 sec^{-1}, and 200 μM and 11.08 sec^{-1}. Similarly, the enzyme from *B. stearothermophilus* involves two sets of values, 3.6 μM and 1.51 sec^{-1}, and 178 μM and 2.77 sec^{-1}. The K_M for tRNA in this case is about 0.6 μM (Figure 6).

It should be noted that the deacylation of Tyr-tRNA is only slightly enhanced by added enzyme. In the presence of 15 μM enzyme (*E. coli*) the $t_{1/2}$ for the hydrolysis of 26 μM [^{14}C]Tyr-tRNA decreases from 21 to 9.6 min.

Hydrolysis of Enzyme Bound Tyrosyl Adenylate. When stripped of ligands after gel filtration, the rate constant for the hydrolysis of the tyrosyl-tRNA synthetase [^{14}C]-Tyr~AMP complex is 4.26×10^{-4} sec^{-1} at 25° and pH 7.78. On the addition of 31 μM tyrosine the rate constant increases to 8.8×10^{-4} sec^{-1}. In the presence of 31 μM tyrosine and 6 mM ATP the [^{14}C]Tyr~AMP is hydrolyzed with a rate constant of 3.5×10^{-3} sec^{-1}. The indirect determination of the hydrolysis rate of the complex in the pres-

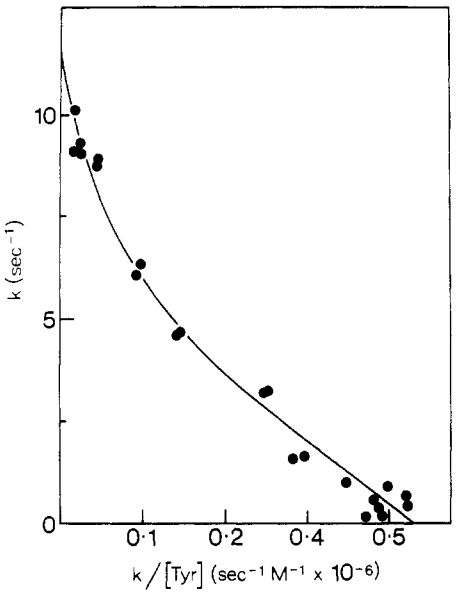

FIGURE 5: k vs. k/[Tyr] for the aminoacylation of tRNA by the tyrosyl-tRNA synthetase from *E. coli*. Curve calculated for biphasic model described in text. (k = initial rate/[enzyme]).

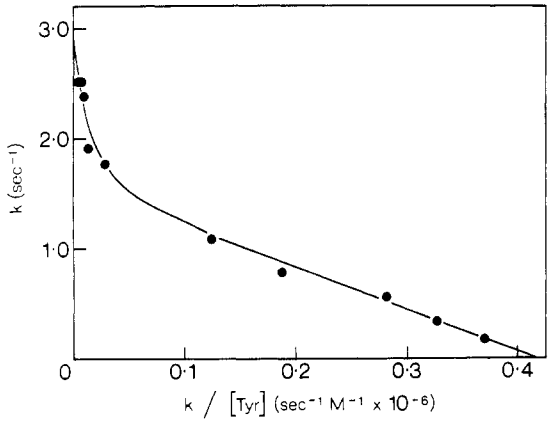

FIGURE 6: k vs. k/[Tyr] for the aminoacylation of tRNA (*E. coli*) by the tyrosyl-tRNA synthetase from *B. stearothermophilus*.

ence of 250 μM tyrosine and 4.6 μM ATP from the rate of depletion of ATP by the method of Fersht (1975) gives a value of 6.5×10^{-3} sec^{-1}.

Discussion

The tyrosyl-tRNA synthetase from *E. coli*, a dimer of mol wt 2 × 47,500, was first isolated by Calendar and Berg (1966). It is generally considered an unstable enzyme and is often isolated in low yield (Chousterman and Chapeville, 1973a). However, it is isolated in high yield in the presence of phenylmethanesulfonyl fluoride and is stable for long periods in the presence of this protease inhibitor.

Stoichiometry of Ligand Binding under Noncatalytic Conditions. Krajewska-Grynkiewicz et al. (1973) and Buonocore and Schlesinger (1972) claim that the tyrosyl-tRNA synthetase from *E. coli* K12 binds one tyrosine (from equilibrium dialysis), one tyrosyl adenylate, and one tRNA (from nitrocellulose filtration) per dimer. Chousterman and Chapeville (1973b) claim that the enzyme from *E. coli* B binds two tyrosyl adenylates (from nonequilibrium gel filtration) and two tRNAs and does not bind tyrosine. They further claim that the above authors' results are incorrect due to their wrongly assaying the protein concentration. Our results, summarized in Table I, on a K12 strain, support the Schlesinger group. Experiments using the active site titration technique of Fersht et al. (1975a), nonequilibrium gel filtration, and nitrocellulose filtration show that only one tyrosyl adenylate is bound per dimer. In agreement with Buonocore and Schlesinger (1972) we find that only one tRNA binds.[1]

Table I: Stoichiometry of Ligand Binding to Tyrosyl-tRNA Synthetases.[a]

Ligand	Added Ligands	Stoichiometry[b] (Dissociation Constant)	
	(a) *E. coli*		
Tyr		1	(24 μM)
Tyr	tRNA	1	(45 μM)
Tyr	tRNA + ATP	1	(15, 200 μM)[c]
Tyr	tRNA, ATP, PP	2	(15, 200 μM)
ATP			(>400 μM)[d]
ATP	tRNA, Tyr, PP		(>200 μM)[d]
tRNA		1	(0.3 μM)
	(b) *B. stearothermophilus*[e]		
Tyr	tRNA, ATP	2	(3.6, 178 μM)[c]
Tyr	tRNA, ATP, PP	2	(3.6, 120 μM)
tRNA		1	
tRNA	Tyr, ATP, PP	1	

[a] pH 7.78, 25° in standard buffer. Determined by equilibrium dialysis except where noted. [b] Rounded to nearest integer. [c] Kinetic determination (K_M values). [d] Equilibrium gel filtration. [e] Further data tabulated by Fersht et al. (1975b).

The question now arises whether or not this partial binding and reactivity is due to the enzyme being partially inactivated. First, the specific activity of the preparation is the highest yet reported (1000 μmol of ATP per 15 min per mg of protein under the conditions defined by Calendar and Berg, 1966). Second, a definitive answer appears to come from the experiment of the binding of tyrosine in the presence and absence of ATP. We have shown that the tyrosyl-tRNA synthetase from *B. stearothermophilus* binds only one tyrosyladenylate per dimer; but on doing so the second site then binds a further mole of ATP and tyrosine. The same is true here: in the absence of other ligands 0.7 mol of tyrosine is bound per dimer; in the presence of ATP and inorganic pyrophosphatase, so that the adenylate is formed in situ, 1.6 mol bind. Similarly, as in the case of the enzyme from *B. stearothermophilus*, the hydrolysis of the enzyme bound tyrosyl adenylate is stimulated by the addition of tyrosine and ATP. Enzymes from both sources must form an enzyme·Tyr~AMP,Tyr,ATP complex.

A further difference between our results and those of Chousterman and Chapeville (1973b) is that whereas they find that a tight complex between the enzyme and ATP which is partially stable on gel filtration, we find, on equi-

[1] A. Pingoud, G. Krauss, D. Boehme, D. Riesner, and G. Maass have informed us that they find two binding sites for $tRNA^{Tyr}$ on the enzyme from *E. coli* MRE 600. One binds tightly, $K_{diss} = 10^{-8}$ M, at pH 6 and the second weakly, $K_{diss} = 5 \times 10^{-7}$ M at pH 6 and 5×10^{-6} M at pH 7.2. At the higher pH and salt concentrations in our experiments the weak site would be beyond the accessible range of tRNA concentration. These results, combined with a dissociation rate constant of 55 sec^{-1} at pH 6 for the second tRNA, suggest that only one tRNA binding site would be detected by nitrocellulose filtration assays. This lends further support to the experimental stoichiometries found by Krajewska-Grynkiewicz et al. (1973).

librium gel filtration, weak binding of ATP; $K_{diss} > 400$ μM. In view of the above results the possibility must be considered that the tyrosyl activating enzymes from *E. coli* B and K12 strains are structurally different although having similar amino acid analyses (Table II). Kondo and Woese (1969) have found differences between the K12 and B strains with the valyl and isoleucyl activating enzymes.

Ligand Binding under Catalytic Conditions. Krajewska-Grynkiewicz et al. (1973) found that the acylation of $tRNA^{Tyr}$ by the synthetase shows biphasic kinetics with respect to tyrosine. We find the same for both the *coli* and *stearothermophilus* enzymes (Figures 5 and 6). This is either due to two genuine sites for tyrosine in the presence of tRNA and ATP or to two competing reaction pathways. The presence of two binding sites was confirmed by equilibrium dialysis using the "equilibrium method" (Figures 1 and 2), i.e., an excess of product, pyrophosphate, is added to reverse the reaction by mass action so that the equilibrium (or steady state) position lies in favor of reagents (Fersht and Renard, 1974; Fersht et al., 1975b). Furthermore, application of this technique to equilibrium gel filtration shows that only one tRNA is bound under these conditions.

Scheme I

$$\begin{array}{ccccc} E & \overset{S}{\rightleftharpoons} & ES & \longrightarrow & EP \longrightarrow \\ & {\scriptstyle K_S} & \Big\| s\,K_S' & & \Big\| s\,K_S'' \\ & & ES_2 & \longrightarrow & ESP \longrightarrow \end{array}$$

Conclusions Concerning Mechanism

It has been shown (Fersht, 1975) that Scheme I may account for half-of-the-sites reactivity and negative cooperativity of substrate binding and provide catalysis. If K_S' is greater than K_S'', but sufficiently small that S may bind at accessible concentrations, then the increase in the strength of binding of one molecule of S as the other reacts to form P may enhance the rate of reaction (case a). Alternatively, if K_S' is so high as to give little occupancy of the site at available substrate concentrations but K_S is relatively small then an unstable intermediate, EP, may be stabilized by the binding of S (case b).

It is known that the enzyme–product complexes (E·AA-tRNA) of the isoleucyl-, leucyl-, valyl-, and phenylalanyl-tRNA synthetases and their aminoacylated tRNAs bind additional molecules of the cognate amino acid and, in some cases, ATP (Yarus and Berg, 1969; Eldred and Schimmel, 1972; Charlier, 1972; Rouget and Chapeville, 1971; Hélène et al., 1971; Bartmann et al., 1974). This appears to be a general phenomenon which satisfies the criterion in the above scheme of forming an "ESP" complex. However, it would seem that case a (with ES = EAA,ATP,tRNA, and ES_2 = EAA_2ATP_2tRNA) is catalytically insignificant as there is (a) no evidence yet for an ES_2 complex for the isoleucyl, leucyl, and valyl enzymes and (b) in this study the binding of the second tyrosine to the E·tRNA·ATP·AA complex occurs at unnaturally high tyrosine concentrations and, in any case only increases the reaction rate by a factor of 2. However, we have shown that the valyl- and tyrosyl-tRNA synthetases from both *E. coli* and *B. stearothermophilus* bind additional substrate molecules to their monoaminoacyl adenylate complexes. In the case of the formation of tyrosyl adenylate by tyrosyl-tRNA synthetase from *B. stearothermophilus* case a was again ruled out but perhaps case b may be important (Fersht et al., 1975). If the half-of-the-sites reactivity is contributing significantly to

Table II: Amino Acid Compositions of Tyrosyl-tRNA Synthetases from *E. coli* B and K12.

	Composition		
	B		
Amino Acid	*a*	*b*	K12
Lys	62	45	57
His	13	15	25
Arg	45	36	42
Cys	15	16	12
Asp	90	83	88.5
Thr	40	41	50
Ser	38	54	39
Glu	111	125	108
Pro	37	31	32
Gly	79	79	69
Ala	77	75	75
Val	51	41	50
Met	18	9	16
Ile	51	36	43
Leu	84	82	82
Tyr	26	22	26
Phe	41	46	48
Try	18	14	10.5

[a] Calendar and Berg (1966). [b] Chousterman and Chapeville (1973a).

catalysis of the overall aminoacylation of tRNA it must, by a process of elimination, be due to the stabilization of an intermediate complex, such as E·AA~AMP·tRNA, e.g.

$$E \cdot tRNA;AA;ATP \rightleftharpoons E \cdot tRNA;AA \sim AMP \rightleftharpoons E \cdot tRNA;AA \sim AMP \longrightarrow \underset{AA,\ ATP}{E \cdot tRNA;AA \sim AMP} \longrightarrow \underset{AA,\ ATP}{E \cdot AA\text{-}tRNA}$$

However, there is insufficient information on this point. We are therefore attempting to obtain further experimental evidence on whether these binding phenomena are of catalytic importance or whether they are just consequences of an as yet unknown phenomenon.

Comment on the Effect of Hydrolysis of Tyr-tRNA on Aminoacylation Kinetics. Aminoacylated tRNAs are unstable with respect to hydrolysis (Schuber and Pinck, 1974, and references therein). The extent of hydrolysis depends of course on the time of incubation and the hydrolysis rate under the given conditions. This may be corrected for by using the procedure given in the Results section. If this procedure is ignored with labile esters serious errors may occur. For example, if a Michaelis–Menten curve is constructed by measuring the reaction rate at different substrate concentrations then the type of error will depend on the experimental technique. If all the solutions are incubated for the same length of time, then, as the extent of hydrolysis is constant for given time, the *shape* of the curve will be correct; the correct value (or values) of K_M will be obtained but V_{max} will be low. If, as is often done, the reactions at lower substrate concentrations are incubated for longer times, the rates of these will be correspondingly underestimated. This will lead to artefactually high values of K_M or in the case of the reactions described here the overlooking of the lower K_M. Similarly, the determination of pH activity profiles may be in error as the lability of the aminoacylated tRNA increases with increasing pH and may also depend on the buffer.

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Demonstration of Two Reaction Pathways for the Aminoacylation of tRNA. Application of the Pulsed Quenched Flow Technique†

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ABSTRACT: A rapid mixing and quenching device is described which operates efficiently in the range of 150 msec to several minutes as well as the usual time scale of 5–150 msec of the conventional apparatus. This has been used to measure the initial rate of acylation of $tRNA^{Tyr}$ by the tyrosyl-tRNA synthetase of *Escherichia coli* during the first turnover of the enzyme, and also the rate constants of the partial reactions of amino acid activation and transfer to the tRNA. It is shown that at saturating concentrations of tRNA the reaction proceeds by a ternary complex mechanism. The rate-determining step is either the aminoacylation process or a step preceding it. At low concentrations of tRNA the reaction proceeds by the stepwise process of formation of tyrosyl adenylate followed by acylation of the tRNA. The rate constants for these partial reactions are faster than that for the ternary complex reaction. But the prior binding of tRNA greatly decreases the rate of tyrosyl adenylate formation. Both pathways are probably important at physiological concentrations. 88% of the tyrosine from the tyrosyl adenylate complex is transferred to tRNA. The presence of added tyrosine and ATP reduces this to 78%. However, the addition of aliquots of ATP to a mixture of enzyme, tyrosine, and a saturating concentration of tRNA (i.e., ternary complex conditions) leads to at least 0.97 mol of tRNA being acylated/mol of ATP hydrolyzed. Trapping experiments show that the 12% of adenylate that is not transferred to tRNA is hydrolyzed on the enzyme rather than expelled into solution.

The mechanism of the aminoacylation of tRNA by aminoacyl-tRNA synthetases has recently been extensively reviewed by Loftfield (1972). He points out that "a consensus has developed in favor of a mechanism in which enzyme reacts, probably first with ATP, then with amino acid to form aminoacyl adenylate; then sequentially to release pyrophosphate, bind tRNA, transfer the aminoacyl group to the tRNA, and, finally, in what might be a rate-determining step, release the esterified tRNA". However, Loftfield himself strongly advocates a mechanism in which tRNA first binds to the enzyme and then reacts with amino acid and ATP in a concerted reaction, i.e., where there is no formation of an aminoacyl adenylate. Some of the evidence in favor of this is that the concentrations of enzymes and tRNA in vivo are such that the enzyme–tRNA complexes

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