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

Alan R. Fersht* and Ross Jakes

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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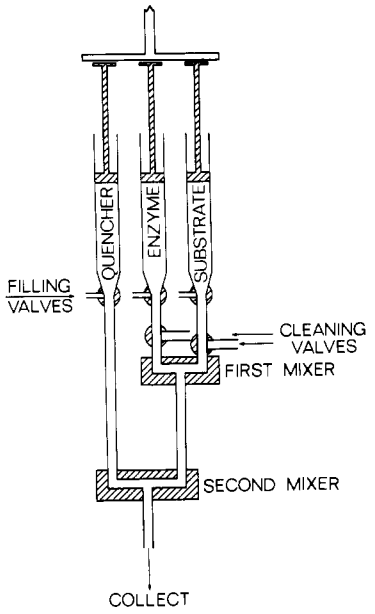

FIGURE 1: Illustration of conventional quenched flow apparatus.

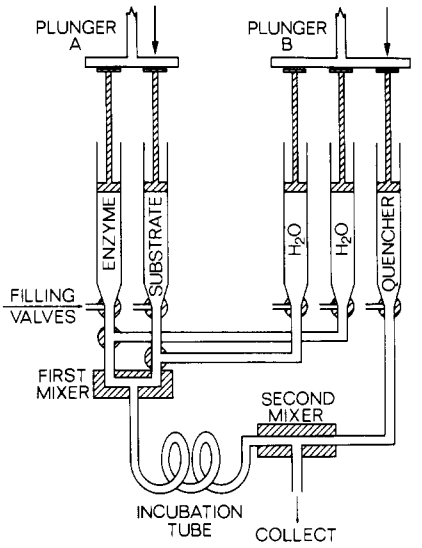

FIGURE 2: Illustration of pulsed quenched flow apparatus.

are the predominant species; certain enzymes under all conditions and other enzymes under special conditions will undergo the pyrophosphate exchange reaction only in the presence of tRNA; and the rate of transfer of the aminoacyl radical from the aminoacyl adenylate complexes to the tRNA is calculated to be too slow to account for the known turnover numbers and also the extent of transfer is too low (Loftfield, 1972; Matsuzaki and Takeda, 1973; Loftfield and Pastuszyn, 1972).

Most of the evidence is indirect. To settle the controversy direct evidence is required concerning the rate of transfer of the aminoacyl moiety from the aminoacyl adenylate complex and the rate constants for the individual chemical steps in the pre-steady-state kinetics for the aminoacylation of tRNA under conditions that are close to physiological. To obtain these data it is necessary to be able to sample the products of the aminoacylation reactions at intervals on the time scale of milliseconds to several seconds. Rapid sampling machines presently available are either limited in use to a too short time range (due to the excessively long flow tubes required for long incubation times, see Lymn and Taylor, 1970) or inefficient in use of valuable protein and time. To overcome these difficulties one of us (A.R.F.) has designed a "pulsed quenched flow" apparatus that can perform the requisite sampling operations relatively efficiently. In this study we describe the apparatus and the results obtained on the aminoacylation of tRNA by the tyrosyl-tRNA synthetase from *Escherichia coli* (K12).

Experimental Section

Materials are described by Fersht (1975) and Jakes and Fersht (1975). The tyrosyl-tRNA synthetase from *E. coli* (EM 20031) was used throughout this study.

Apparatus and Kinetic Techniques. Unless otherwise stated all kinetic studies were performed at 25.0 ± 0.1° in a standard buffer of 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.

Quenched Flow and Pulsed Quenched Flow Apparatus. Figure 1 is a diagrammatic illustration of the conventional quenched flow mode (cf. Lymn and Taylor, 1970). Enzyme and substrate are incubated, as shown, in the thermostated syringes. They are then mixed as the plunger, driven by compressed air, drives them through the first mixing chamber, and then age on passing to the second mixer when quenching occurs on mixing with the trichloroacetic or perchloric acid from the quenching syringe. The travel of the plunger is monitored as an oscilloscope trace of the voltage from a linear potentiometer which is attached to the pushing bar. If the volume expelled from the "enzyme" and "substrate" syringes is V, the volume between two mixers is v and the total time of travel, monitored on the oscilloscope, is T, then the age of the mixture on quenching, t, is given by

$$t = Tv/V$$

By varying the driving pressure (13–60 psi) and using different lengths of tubing between the two mixing chambers (55 and 200 μl) the range of the instrument is 5–140 msec. A volume of 292 μl is expelled from each syringe.

The pulsed-quenched flow mode is illustrated in Figure 2. Again the motion of either plunger A or B is monitored on an oscilloscope from the voltage of a linear potentiometer which is attached to the pushing bar of each. On actuation of the electronic valve that controls the compressed air supply to plunger A the storage oscilloscope is triggered, the plunger mixes enzyme and substrate from the thermostated syringes (292 μl from each), and the resultant mixture ages in the thermostated incubation tube. After a predetermined time, 150 msec to 100 sec or greater, the compressed air supply to plunger B is electronically activated. As this descends distilled water (1.13 ml total) from B drives the enzyme–substrate mixture out of the incubation tube through the second mixer where it is quenched by the acid (0.565 ml) which is simultaneously driven through by B. The residence time of the solution in the incubation tube is simply read from the oscilloscope screen. (As the distilled water is driven through the apparatus from above the mixing chamber a small fraction of solution does not incubate in the tube apart from the travel time through (about 145 msec). A correction is made for this.)

After the run the machine has, in effect, cleaned itself with the pulse of distilled water. However, as a precaution, 10 ml of distilled water is flushed through the tubes from the "H_2O" syringes and through the two mixing chambers and the interconnecting incubation tube. The same procedure is used to clean the apparatus in the conventional

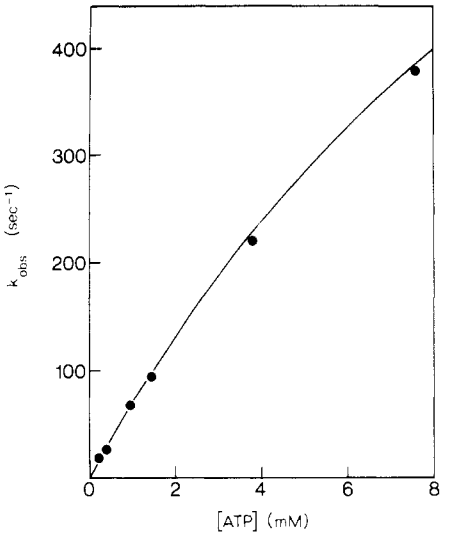

FIGURE 3: Rate constants for the formation of tyrosyl adenylate from the tyrosyl-tRNA synthetase (0.7 μM), tyrosine (115 μM), and varying concentrations of ATP using the stopped-flow spectrofluorimeter.

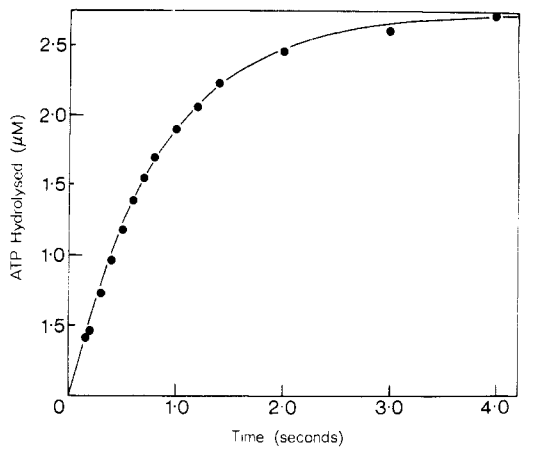

FIGURE 4: Formation of tyrosyl adenylate on mixing equal volumes of tyrosyl-tRNA synthetase (4.9 μM) and ATP (30.5 μM) in presence of tyrosine (115 μM) and inorganic pyrophosphatase using the pulsed quenched flow apparatus. Solid curve calculated for a rate constant of 1.18 sec^{-1}.

quenched flow mode. The distilled water is left in the apparatus before each run to maintain a constant back pressure during the travel of the plunger.

In either mode it is possible to construct a time course of 20 quenchings during 1 hr.

Rate of Formation of Tyrosyl Adenylate. (a) Stopped-Flow Fluorescence (Fersht et al., 1975a,b). One syringe of the stopped-flow spectrofluorimeter contained 0.9 μM enzyme and 230 μM tyrosine in the standard buffer; the other syringe contained ATP (7 μM–15 mM) in the same buffer. At low concentrations of ATP inorganic pyrophosphatase was added to prevent any back reaction. The decrease in fluorescence on mixing the reagents was monitored on a storage oscilloscope and also, by using a DASAR system, on punched tape.

(b) Pulsed Quenched-Flow. One syringe contained 4.92 μM enzyme, 230 μM tyrosine, and 3 units/ml of inorganic pyrophosphatase in the standard buffer. The other syringe contained 30.5 μM [γ-^{32}P]ATP in the same buffer. The quenching syringe contained 7% perchloric acid. The quenched effluent was collected in tubes containing 150 μl of a solution of 1.75 M sodium formate and 2.5 M KOH (to precipitate the perchlorate and to raise the pH of the effluent to 3.5); 10 μl of the effluent was spotted onto PEI sheets (15 × 4 cm), which had previously been treated with ATP and AMP to act as markers, and developed in 0.5 M phosphate buffer (pH 3.5) (Cashel et al., 1969). After drying the sheets were examined under a short wavelength uv lamp, the regions containing ATP and AMP (phosphate and AMP run with the ion front, ATP has a low R_F) were cut out, and the radioactivity was assayed using a water-miscible scintillant.

Initial Rate of Acylation of tRNA. The pulsed quenched flow mode was used. One syringe contained enzyme and tRNA^{Tyr} in the standard buffer, the other syringe contained [^{14}C]Tyr, ATP in the standard buffer (with or without inorganic pyrophosphatase) as indicated in the legends to the figures. The quenching syringe contained 10% trichloroacetic acid. The effluent was collected, filtered onto nitrocellulose disks, washed, and dried and the radioactivity assayed with the scintillation counter using the toluene based scintillant (Fersht et al., 1975a). Some 10–20 samples were quenched in the range 150–800 msec.

Initial Rate of ATP Hydrolysis on Acylation of tRNA. Again the pulsed quenched flow mode was used but the quenching syringe contained 7% perchloric acid. One syringe contained enzyme (38 μM), tRNA (60 μM), and inorganic pyrophosphatase (10 units/ml) in the standard buffers. The other syringe contained tRNA (60 μM), tyrosine (100 μM), and [γ-^{32}P]ATP (1.08 mM) in the same buffer. The quenched effluent was collected in tubes containing 150 μl of 2.5 M KOH and 1.75 M sodium formate as before. In order to ensure complete hydrolysis of the pyrophosphate before assaying, 20 μl of a solution of Tris-Cl (1.4 M, pH 7.78) and $MgCl_2$ (100 mM) were added to 100 μl of effluent; 1 μl (1 unit) of inorganic pyrophosphatase was added, the solution was left for 1 min at room temperature, and the pH was then lowered by the addition of 10 μl of 4 N formic acid. The ratio of inorganic phosphate to ATP was then assayed using PEI sheets as before.

Rate of Transfer of Tyrosine from E·Tyr~AMP to tRNA. The tyrosyl-tRNA synthetase [^{14}C]tyrosyl adenylate was prepared by incubating the enzyme with ATP, [^{14}C]Tyr (96 Ci/mol), and inorganic pyrophosphatase and isolating by gel filtration in 5 mM bis-tris (pH 5.85), 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. One syringe of the quenched flow apparatus contained the adenylate (1.0 μM) in this buffer, the other contained 12 μM tRNA^{Tyr} in either a buffer of 288 mM Tris-Cl (pH 7.78) and 10 mM $MgCl_2$ or in the same buffer plus 250 μM tyrosine and 4 mM ATP. In another experiment, one syringe contained ~2.5 μM enzyme incubated in 135 μM [^{14}C]Tyr (37 Ci/mol), 4 mM ATP, and 1 unit/ml of inorganic pyrophosphatase in the standard (pH 7.78) buffer and the other syringe contained 12 μM tRNA^{Tyr} in that buffer and also 1.5 mM tyrosine. Time courses of the reaction were constructed (6–140 msec) by mixing, quenching with 10% trichloroacetic acid, and collecting on nitrocellulose filters. In the first two experiments aliquots of the adenylate solution were filtered through nitrocellulose filters to monitor the hydrolysis under these conditions ($t_{1/2}$ = 4.74 hr). The concentration of adenylate was then corrected for hydrolysis.

Extent of Acyl Transfer and Efficiency of ATP Hydrolysis. The efficiency of the precipitation assay for [^{14}C]Tyr-tRNA was first checked by comparing the number of counts precipitated onto the nitrocellulose filter as com-

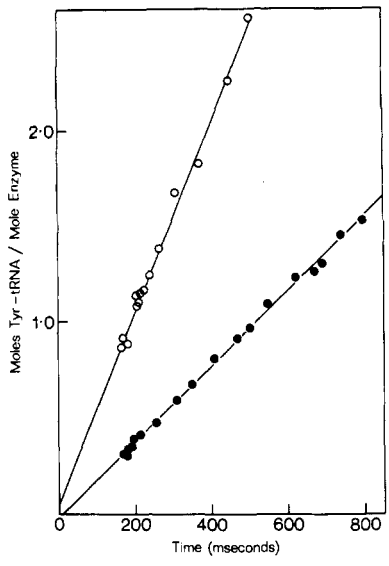

FIGURE 5: Initial rate of charging of $tRNA^{Tyr}$ using [^{14}C]Tyr and the pulsed quenched flow apparatus. Enzyme was preincubated with tRNA in one syringe and mixed with tyrosine and ATP from the other syringe to give the following final concentrations: (○) 0.97 μM tyrosyl-tRNA synthetase, 4.65 μM $tRNA^{Tyr}$ (1000 pmol/A_{260}), 2 mM ATP, and 55 μM [^{14}C]Tyr; (●) 2.6 μM enzyme, 10 μM $tRNA^{Tyr}$ (274 pmol/A_{260}), 524 μM ATP, 55 μM [^{14}C]Tyr, and inorganic pyrophosphatase (5 units/ml).

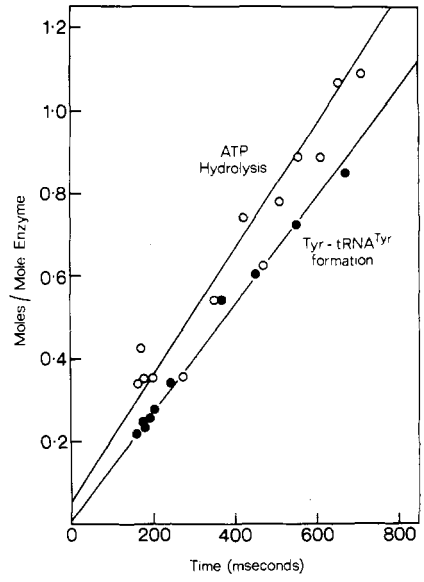

FIGURE 6: Initial rate of charging of $tRNA^{Tyr}$ (monitored by [^{14}C]Tyr incorporation) and simultaneous rate of adenylate formation (monitored by ATP hydrolysis). Enzyme was preincubated with tRNA and mixed with ATP and tyrosine to give the following concentrations: 60 μM $tRNA^{Tyr}$ (384 pmol/A_{280}), 19 μM enzyme, 55 μM tyrosine, 538 μM ATP, and inorganic pyrophosphatase; (○) [^{14}C]Tyr incorporation into tRNA; (○) hydrolysis of [γ-^{32}P]ATP.

pared with the total number of counts. Using 25-μl aliquots of 12 or 19 μM tRNA and precipitating with cold 5% trichloroacetic acid the assay was found to be 98.5% efficient.

(a) Transfer from Preformed E·[^{14}C]Tyr~AMP. The complex was prepared and desalted on a G-25 (fine) column equilibrated with 5 mM bis-tris (pH 5.85), 10 mM $MgCl_2$, and 10 mM mercaptoethanol as previously described. Aliquots were added to tRNA solutions under various conditions, incubated (for a few seconds at pH 7.78 or 1–2 min at pH 5.85), and quenched with trichloroacetic acid and the extent of acylation was determined by collecting the filtrate on nitrocellulose filters and monitoring the radioactivity. Each experiment was performed in triplicate.

(b) Addition of Aliquots of ATP to E·tRNA[^{14}C]Tyr. Aliquots of ATP, containing from 0.97 to 9.7 nmol, were added to solutions (180 μl) containing 10 nmol of $tRNA^{Tyr}$, 1.45 nmol of tyrosyl-tRNA synthetase, 130 nmol of [^{14}C]Tyr (20 Ci/mol), and 5 units inorganic pyrophosphatase in 40 mM Tris-Cl (pH 7.78), 5.6 mM $MgCl_2$, 2.8 mM mercaptoethanol, and 0.03 mM phenylmethanesulfonyl fluoride; 10-μl aliquots were taken at 0.5- or 1-min intervals for 6 min and quenched with trichloroacetic acid, and the [^{14}C]Tyr-tRNA was collected and washed on nitrocellulose filters. The concentration and purity of each reagent were checked by standard procedures ([^{14}C]Tyr = 96.5% radiochemically pure; tyrosine, recrystallized; ATP, 99% pure).

The rate of hydrolysis under these conditions was also checked after replacing the tRNA by [^{14}C]Tyr-tRNA and the ATP by AMP ($t_{1/2}$ = 27 min).

Results

Tyrosyl Adenylate Formation in the Absence of tRNA. When the enzyme is incubated in 230 μM tyrosine and then mixed with ATP there is a 6% decrease in the fluorescence of the tryptophans. The rate constant for this change increases with increasing ATP concentration. Saturation kinetics appear to hold but the observed value of K_M, ~18 mM, is too high for reliable determination. This is associated with a k_{cat} of about 1.3×10^3 sec^{-1}. k_{cat}/K_M is 7.3×10^4 sec^{-1} M^{-1} (see Figure 3). This process is shown to correspond to aminoacyl adenylate formation from the pulsed quenched flow experiment (Figure 4). 15.25 μM ATP reacts with the enzyme to form 1 mol of adenylate with a rate constant of 1.18 sec^{-1}. The rate constant for the fluorescence change is 1.11 sec^{-1} at this concentration.

Initial Rate of Acylation of tRNA. It is seen in Figures 5 and 6 that there is no "burst" of Tyr-tRNA formation. In the presence of 2 mM ATP and 55 μM tyrosine an initial concentration of 4.65 μM $tRNA^{Tyr}$ is charged with a rate constant of 4.94 sec^{-1} (burst = 0.06 ± 0.04 mol of Tyr-tRNA/mol of enzyme). In the presence of 520 μM ATP and 55 μM tyrosine this drops to 1.96 sec^{-1} (burst = 0.01 ± 0.01) for 10 μM tRNA reacting with 2.6 μM enzyme. These rate constants are considerably lower than for the rate of tyrosyl adenylate formation in the absence of tRNA, values of >130 and >40 sec^{-1}, respectively, being expected.

The initial rate of hydrolysis of ATP is shown in Figure 6. If there is an initial burst it is very small (0.057 ± 0.052 mol of ATP/mol of enzyme for 538 μM ATP, 55 μM tyrosine, 18.9 μM enzyme, and 60 μM tRNA). These experiments are close to the limits of detection and an error of ±0.1 or so is possible. It is clear that there is no large burst of ~1 ATP/mol of enzyme.

Rate of Acyl Transfer from the Adenylate to tRNA. The results of the quenched flow experiments are illustrated in Figure 7. On mixing 1 μM adenylate complex, incubated in a dilute pH 5.85 buffer, with 12 μM tRNA, in a concentrated pH 7.78 buffer, there is a relatively rapid transfer of tyrosine to the tRNA with a rate constant of 35.2 sec^{-1}. On the addition of tyrosine (250 μM) and ATP (4 mM) the rate constant decreases slightly to 28.3 sec^{-1} and the efficiency of transfer also slightly, but significantly, decreases. When the adenylate is prepared in situ at pH 7.78 by incubation with excess [^{14}C]Tyr and ATP and then mixed with tRNA (in the presence of a large excess of "cold" tyrosine to minimize the incorporation of [^{14}C]Tyr during the

Table I: Efficiency of Acyl Transfer from Tyrosyl-tRNA Synthetase [^{14}C]Tyr~AMP[a] to tRNA^{Tyr}.

Final pH	[E·Tyr~AMP] (μM)	[tRNA] (μM)	Added Reagents	Extent of Transfer (%)
5.85	2.8	24		86
5.85	2.8	24	Enzyme[b] (5 μM)	81
7.78	1.1	6.4		88.5
7.78	0.8	14	Enzyme (2 μM)	87
7.78	0.32	1.8	Enzyme (4.6 μM)	84
7.78	1.9	7.7		88.5
7.78	1.9	7.7	Tyr (480 μM) ATP (3.8 mM)	78

[a] In pH 5.85 bis-tris. [b] Tyrosyl-tRNA synthetase.

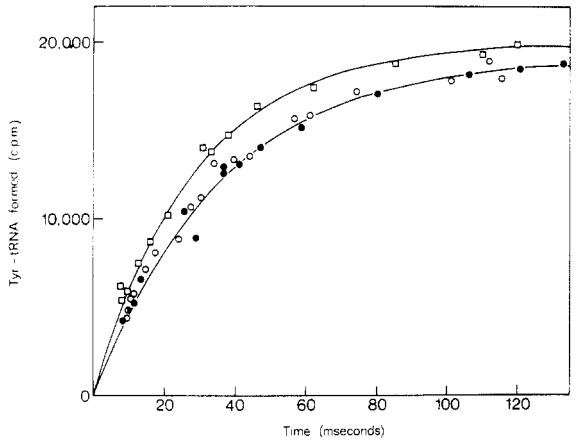

FIGURE 7: Transfer of [^{14}C]Tyr from tyrosyl-tRNA synthetase [^{14}C]Tyr·AMP to tRNA determined using the quenched flow apparatus as described in the text. (□) Ligand free tyrosyl adenylate complex (0.5 μM) incubated at pH 6 and jumped to pH 7.78 on mixing with tRNA (12 μM); (●) as above but tRNA solution contains ATP and tyrosine; (○) tyrosyl adenylate formed in situ by incubating enzyme with ATP, [^{14}C]Tyr, and pyrophosphatase at pH 7.78. Upper curve calculated for a rate constant of 35.2 sec^{-1}, lower for 28.3 sec^{-1}.

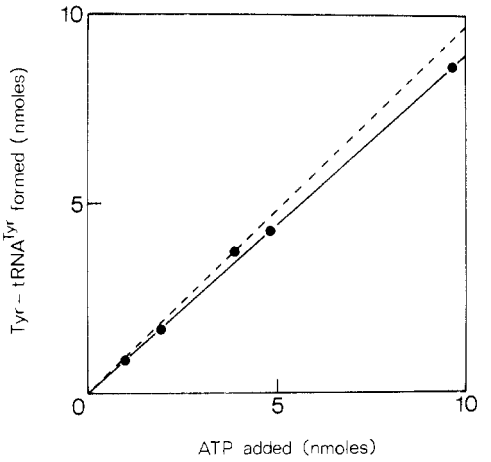

FIGURE 8: Tyr-tRNA formed on addition of ATP to a mixture (180 μl) of tRNA (55 μM), tyrosyl-tRNA synthetase (8 μM), tyrosine (722 μM), and inorganic pyrophosphatase. (—) Observed maximum value (slope = 0.894). (- - -) (slope = 0.97) observed maximum value corrected for hydrolysis.

steady-state turnover) there appears to be identical results to when incubation is at pH 5.85.

Acyl Transfer from Adenylate to tRNA^{Tyr} and Stoichiometry of ATP Hydrolysis. The extent of transfer of [^{14}C]Tyr from the E·[^{14}C]Tyr~AMP complex to tRNA^{Tyr} was measured under various conditions for three purposes: (a) to determine the absolute efficiency of transfer (to compare with the observed hydrolysis of ATP for the overall reaction); (b) to see if added tyrosyl-tRNA synthetase can increase the efficiency of transfer (by trapping any tyrosyl adenylate that becomes free in solution; and (c) to see if adding tyrosine and ATP, which may bind to the second site, affects the efficiency.

The results are summarized in the table. (a) The transfer is up to 88.5% efficient at pH 7.78; (b) added enzyme, if anything, lowers the efficiency of transfer; (c) added tyrosine and ATP cause a 12% decrease in transfer efficiency.

The stoichiometry of ATP hydrolysis in the overall reaction was determined by adding aliquots of ATP to a mixture of enzyme, tRNA, and [^{14}C]Tyr and determining the number of moles of tRNA charged on total hydrolysis of the ATP. The amount of tRNA acylated reaches a maximum after 1–4 min depending on the amount added and then decreases due to hydrolysis. The observed maximum values are plotted in Figure 8. At this point 0.894 mol of tRNA are charged for every mol of ATP added. Correcting the observed values for hydrolysis ($t_{1/2}$ = 27 min) increases this value to 0.97 mol/mol. Any systematic error in the experiments, e.g., ATPase activity, incomplete reaction, purity of reagents, etc., will tend to underestimate the efficiency of tRNA acylation. This process is at least 97% efficient relative to ATP hydrolysis, significantly higher than observed in the acyl transfer experiments (78% in the presence of ATP and tyrosine).

Estimation of Dissociation Rate Constant of Charged tRNA from the Enzyme. Limits for this value may be estimated from the size of the "bursts" for the charging reactions illustrated in Figures 5 and 6, and the subsequent turnover numbers. Values of >100 sec^{-1}, >50 sec^{-1}, and >80 sec^{-1} are calculated. Clearly at saturating concentrations of reagents this step is not rate limiting since it is at least five times faster than the turnover number (11 sec^{-1}).

Finally, it should be noted from control experiments that (a) the specific activity of the tRNA does not appear to alter the kinetics and (b) the addition of tRNA to the pyrophosphate exchange assay causes approximately the expected inhibition at the concentrations used. This latter result is not open to a simple interpretation since unpublished data from this laboratory indicate that the addition of pyrophosphate (as in the pyrophosphate exchange assay) depresses the rate constant for adenylate formation.

Discussion

Pulsed-Quenched Flow Apparatus. The novel feature of the pulsed-quenched flow machine used here is that extended incubation times may be attained by mixing enzyme and substrate in an incubation tube and then, after a predetermined time, flushing them with a pulse of water into a mixing chamber and chemical quencher. The apparatus is very

economical in protein solution, rapid to use due to the automatic cleaning procedure, and accurate. It is similarly economical in the conventional quenched flow mode.

Using this we have been able to obtain otherwise inaccessible data on the aminoacylation of tRNA.

Mechanism. The questions we are attempting to answer are those raised by Loftfield, what is the reaction pathway and what is the rate-determining step? It will be shown later that these depend on substrate concentrations. Now the concentrations of ATP and tyrosine in the *E. coli* cell are about 0.5–2.5 m*M* and 40 μM, respectively (see Blanquet et al., 1973, for the method of calculation and references), while the concentration of aminoacyl-tRNA synthetase is expected to be in the μM region and that of the tRNA somewhat greater (Calendar and Berg, 1966; Yarus and Berg, 1969; Jacobson, 1969). These will be our reference points. Noting the equilibrium binding constants determined in the accompanying paper (Jakes and Fersht, 1975) it is expected that the enzyme exists under these conditions as mainly the enzyme-tRNA complex.

(1) The Rate-Determining Step. If, as is often stated (Loftfield, 1972) the rate-limiting step in the charging of tRNA is the diffusion of the aminoacylated tRNA from the enzyme, then on mixing a solution of enzyme and tRNA with ATP and tyrosine there should then be an initial fast burst of aminoacylation followed by a slower steady state turnover as found for the isoleucyl enzyme at pH 6 and 3° (Eldred and Schimmel, 1972a). It is seen in Figure 5 that using concentrations that approximately span the physiological values, there is no burst; the steady state is attained immediately. The first conclusion is that the rate-determining step precedes the aminoacylation reaction and is certainly not the diffusion controlled dissociation of the enzyme–AA-tRNA complex. (This result may be extrapolated to saturating reagent concentrations.)

(2) The Chemical Pathway. The charging reaction may be artificially dissected into the two-stage process

$$\text{ATP} + \text{Tyr} + \text{E} \xrightarrow{k_1} \text{E·Tyr} \sim \text{AMP} + \text{PP}$$

$$\text{E·Tyr} \sim \text{AMP} + \text{tRNA} \xrightarrow{k_2} \text{E} + \text{Tyr-tRNA} + \text{AMP}$$

by (a) measuring the rate constant k_1 in the absence of tRNA and (b) preforming the aminoacyl adenylate complex and then mixing with tRNA. It is seen in Figures 3 and 6 that these two reactions are relatively fast, e.g., $k_1 \sim 40$ sec^{-1} for 0.5 m*M* ATP, and $k_2 \sim 30$ sec^{-1} for 12 μM tRNA. The *steady-state* turnover rate under these conditions is about 2 sec^{-1} (Figures 4 and 5). However, in the steady-state reaction under these conditions the bulk of the enzyme is not free but liganded with tRNA. The relative slowness of the steady-state rate is not due to a step subsequent to aminoacylation being rate determining as we have already ruled this out (see above); it is not due to the accumulation and slow decomposition of an E·Tyr ~ AMP·tRNA complex since the transfer step k_2 is fast. The second conclusion is that the prior binding of tRNA to the enzyme causes the aminoacyladenylation reaction to slow down to such an extent that it either becomes rate determining or gives away to an alternative concerted pathway, as proposed by Loftfield. In order not to prejudge the chemical nature of the second pathway we shall describe it as the ternary complex mechanism.

However, even though the bulk of the enzyme is liganded with tRNA some fraction remains free. Supposing the dissociation constant for this interaction is about 0.3 μM and the tRNA concentration is about 10 μM then only 3% of the enzyme is free of tRNA, and able to charge tRNA by the stepwise route. But the rate constant for this pathway is faster by a factor of 10 or so than when the enzyme is initially bound with the tRNA. The stepwise mechanism under these conditions would contribute a rate constant of 0.5 sec^{-1}, i.e., about 25% of a steady-state rate of 2 sec^{-1}. Clearly, at high concentrations of tRNA more enzyme will be liganded and the ternary complex mechanism will be dominant; at low concentrations the stepwise mechanism will be more important. (This could cause odd effects in the determination of the K_M for tRNA.) The third conclusion is then that under physiological conditions two reaction pathways are available for tRNA charging.

Nature of the Ternary Complex Mechanism. Under the conditions of high tRNA concentrations to suppress the stepwise mechanism we found no significant burst of pyrophosphate release from ATP that could indicate the prior formation of an aminoacyl adenylate complex. This is not to say that one does not form; if the formation rate constant under our conditions is 2 sec^{-1} and the transfer rate (k_2 in the stepwise scheme) is 30 sec^{-1} then a burst of only 0.004 mol of pyrophosphate/mol of enzyme is expected. Also on theoretical grounds it is catalytically disadvantageous for intermediates to accumulate, but rather they should be present in low steady-state concentrations (Fersht, 1974). The ternary complex mechanism is either then a concerted process as proposed by Loftfield or a stepwise mechanism in which the transfer step is relatively fast. Experimentally these situations are always difficult to resolve. This is especially so here as an alternative stepwise pathway has been demonstrated and this will obscure the issue if attempts are made to isolate under extreme conditions the hypothetical intermediate in the ternary complex mechanism.

Interrelationship between Specificity and Mechanism. There is insufficient difference between isoleucine and valine for the isoleucyl-tRNA synthetase to distinguish between the two sufficiently to account for the fidelity of protein synthesis if just a single step mechanism is involved. Various verification mechanisms have been found: addition of tRNA^{Ile} to the isoleucyl synthetase valyl adenylate complex causes its quantitative hydrolysis rather than transfer (Baldwin and Berg, 1966); Val-tRNA^{Ile} and other misacylated tRNAs are deacylated by their cognate aminoacyl-tRNA synthetases (Eldred and Schimmel, 1972b; Yarus, 1972). However, Bonnet and Ebel (1974) assert that the deacylation mechanism of the misacylated tRNA is inadequate to account for the fidelity of protein synthesis. Since there is no way of increasing specificity by having interacting active sites (on subunits, etc.) (Fersht, 1975) then, if Bonnet and Ebel are correct, the concerted mechanism must be ruled out. Without this correction procedure a mechanism involving consecutive chemical steps is required (Hopfield, 1974). Conversely, a concerted charging mechanism implies a subsequent deacylation mechanism.

Relationship to Mechanism and Specificity to Efficiency of Acyl Transfer. The efficiency of transfer of the tyrosyl moiety from the preformed adenylate to tRNA varies from 78 to 88.5% depending on conditions. However, when aliquots of ATP are added under "ternary complex conditions" to a solution of enzyme, tyrosine, and a high concentration of tRNA at least 0.97 mol of tRNA are charged for every mol of ATP hydrolyzed (Figure 7). At first sight this would indicate a fundamental difference between the two

mechanisms. However, the possibility must be considered that there are two binding sites for the tRNA and that in the stepwise mechanism a fraction may bind to the "nonproductive" site and cause hydrolysis of the aminoacyl adenylate. It should be noted that the incomplete transfer of the tyrosyl from the complex is not due to the tyrosyl adenylate being expelled into solution on, or prior to, the binding of tRNA since the addition of excess enzyme to the solution to trap any such species does not increase the efficiency of transfer. This has also been shown for the isoleucyl enzyme by Baldwin and Berg (1966) using a different technique. This appears to rule out the "kinetic proof reading" mechanism proposed by Hopfield (1974) in which discrimination between correct and incorrect aminoacyl adenylate complexes results from differential rates of dissociation. Instead, a specific hydrolytic site on the enzyme is implied. The incomplete transfer of amino acid in the stepwise mechanism may be important in specificity. If there are competing pathways for the transfer of the amino acid moiety to tRNA and water then greater discrimination is possible if the rates of the two are delicately balanced in the case of a correctly formed complex. Then for an incorrect complex the difference in binding energy and fit may displace the ratio far in favor of hydrolysis.

Relationship to Published Data. Analysis of steady-state kinetics by the method of Cleland (1963) has shown that the prolyl enzyme from *E. coli* and the threonyl from rat liver (Papas and Mehler, 1971; Allende et al., 1970) acylate tRNA by the stepwise mechanism. On the other hand, the arginyl ligase from *E. coli* and presumably the glutamyl and glutaminyl (Papas and Peterkofsky, 1972; Ravel et al., 1965) involve a mechanism requiring simultaneous binding of amino acid, ATP, and tRNA. The phenylalanyl-enzymes from *E. coli* and yeast may represent a mixture of mechanisms (Santi et al., 1971; Berthe et al., 1974). As we have shown by direct means that the tyrosyl-enzyme may acylate tRNA by both mechanisms it is reasonable to expect that there is no unique mechanism as argued by Loftfield (1972).

The reason why there are two mechanisms is not clear. Perhaps this is related to specificity. Also the nature of the ternary complex mechanism is not yet known. We are continuing our investigations of these problems.

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