Catalytic Mechanism of Glutamyl-tRNA Synthetase from *Escherichia coli*. Reaction Pathway in the Aminoacylation of $tRNA^{Glu}$ †

Daniel Kern* and Jacques Lapointe

ABSTRACT: Glutamyl-tRNA synthetase does not form an isolable aminoacyl·adenylate complex in the presence of adenosine 5′-triphosphate (ATP) and amino acid and does not catalyze the ^{32}P-labeled inorganic pyrophosphate ([^{32}P]-PP_i)–ATP exchange in the absence of $tRNA^{Glu}$. These particular properties suggest a priori the existence of a concerted aminoacylation mechanism for this synthetase. We show (1) that $tRNA^{Glu}$ stimulates the isotope exchange more efficiently than Glu-$tRNA^{Glu}$ plus adenosine 5′-monophosphate (AMP) and (2) that the isotope exchange occurs faster than the tRNA charging step and its reversal. These results indicate (1) that Glu-$tRNA^{Glu}$ and AMP are not directly involved in the isotope exchange and (2) that neither the tRNA charging step nor its reversal is involved in the isotope exchange. In addition, glutamyl-tRNA synthetase catalyzes an equilibrated AMP-dependent and PP_i-independent Glu-$tRNA^{Glu}$ deacylation reaction; the initial rate of this reaction equals the rate of the overall reversal of tRNA charging, indicating that the latter reaction occurs via a two-step pathway, whose first step is rate determining. The AMP-dependent and PP_i-independent Glu-$tRNA^{Glu}$ deacylation gives rise to the formation of an intermediate able to transfer further glutamate to $tRNA^{Glu}$ in the absence of ATP. The glutamyl-tRNA synthetase complex involving AMP and glutamate in a 1:1 stoichiometry could be isolated by gel filtration of the reaction mixture containing the synthetase, Glu-$tRNA^{Glu}$, and AMP, either in AMP equilibrium conditions or after stabilization of the complex by addition of a large excess of $tRNA^{Glu}$-CCA to the mixture of reactants.

The mechanism of aminoacylation of tRNA by aminoacyl-tRNA synthetases is a subject of large controversy. Because 17 of the 20 synthetases catalyze an amino acid dependent [^{32}P]PP_i–ATP[1] exchange in the absence of their tRNA and for several of these enzymes stable enzyme·adenylate complexes could be isolated (able to transfer further their activated amino acid to the tRNA), a two-step aminoacylation mechanism was first proposed [for general reviews, see Kisselev & Favorova (1974), Söll & Schimmel (1974), Kalousek & Konigsberg (1975), Goddard (1977), and Ofengand (1977)]. This scheme implied the involvement of the enzyme·adenylate intermediate in the overall tRNA charging process.

$$E + aa + ATP \overset{MgCl_2}{\rightleftharpoons} E{\cdot}AMP{\sim}aa + PP_i$$

$$E{\cdot}AMP{\sim}aa + tRNA \rightleftharpoons aatRNA + AMP + E \quad (1)$$

However, arginyl-, glutamyl-, and glutaminyl-tRNA synthetases of various organisms are unable to catalyze the [^{32}P]-PP_i–ATP exchange in the absence of their tRNA (see the previously mentioned general reviews). In addition, the usual approaches did not allow the isolation of an enzyme·adenylate complex for these synthetases. These particular behaviors were the major arguments for the proposition, at least for these three synthetases, of a one-step mechanism. According to this scheme, the three substrates must be bound to the enzyme in order to initiate a concerted catalytic process (Loftfield, 1972):

$$E + aa + ATP + tRNA \overset{MgCl_2}{\rightleftharpoons} aatRNA + AMP + PP_i + E \quad (2)$$

However, most of the mechanistic studies carried out during the last years strengthened essentially the stepwise pathway for the tRNA aminoacylation reactions. Using the quenched-flow method, Fersht and co-workers obtained results supporting the aminoacyladenylate pathway (eq 1) in the aminoacylation reaction catalyzed by several synthetases that form an isolable aminoacyladenylate. In valyl-, isoleucyl-, tyrosyl-, phenylalanyl-, and methionyl-tRNA systems this intermediate is formed rapidly enough and also reacts rapidly enough to be on the aminoacylation pathway (Fersht & Jakes, 1975; Fersht & Kaethner, 1976; Fasiolo & Fersht, 1978; Mulvey & Fersht, 1978). More recently, Fersht et al. (1978) gave arguments for the existence of a two-step pathway for the yeast arginyl-tRNA synthetase, a representative enzyme of the class of synthetases requiring the tRNA to catalyze the [^{32}P]PP_i–ATP exchange. This synthetase catalyzes an AMP-dependent and PP_i-independent deacylation of Arg-$tRNA^{Arg}$ with a rate similar to the rate of the overall reversal of $tRNA^{Arg}$ charging, suggesting that this last reaction, and thus also the $tRNA^{Arg}$ charging, occurs stepwise. But, as pointed out by the authors, their results did not exclude definitively the concerted mechanism for this synthetase. Mehler and co-workers gave arguments for a two-step aminoacylation pathway in the *Escherichia coli* arginylation and valylation systems. Investigating the pH effect on the partial reactions catalyzed by *E. coli* arginyl-tRNA synthetase, Lui et al. (1978) demonstrated (1) that different chemical groups of the synthetase participate in the rate-limiting steps of pyrophosphorolysis of arginyladenylate and transfer of arginine to $tRNA^{Arg}$ and (2) that these partial reactions and their reversals have properties consistent with their participation in the overall $tRNA^{Arg}$ charging. On the other hand, Midelfort

† From the Département de Biochimie, Faculté des Sciences et de Génie, Université Laval, Quebec G1K 7P4, Canada (J.L.), and the Institut de Biologie Moléculaire et Cellulaire du CNRS, Laboratoire de Biochimie, 67084 Strasbourg Cédex, France (D.K.). *Received October 17, 1979.* This work was supported by a grant from the National Research Council of Canada to J.L. and by grants from the Délégation Générale à la Recherche Scientifique et Technique and the Centre National de La Recherche Scientifique.

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Glu-$tRNA^{Glu}$, $tRNA^{Glu}$ aminoacylated with glutamate; $tRNA^{Glu}$-CCA, $tRNA^{Glu}$ without the 3′ OH–CCA end; PP_i, inorganic pyrophosphate; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate.

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et al. (1975) showed that the effects of substrate concentrations on the rates of exchange of isotopically labeled substrates at the chemical equilibrium of the *E. coli* $tRNA^{Val}$ charging system were in agreement with a stepwise mechanism for this synthetase. Such a mechanism was already convincingly proposed in the early 1970s by Schimmel and co-workers for $tRNA^{Ile}$ charging. These authors showed that (1) Ile~AMP promotes the $tRNA^{Ile}$ charging as fast as isoleucine and ATP and (2) Ile~AMP promotes the dissociation of the nascent Ile-$tRNA^{Ile}$ at the steady state of $tRNA^{Ile}$ charging (Eldred & Schimmel, 1972; Söll & Schimmel, 1974).

We investigated the mechanism of the *E. coli* glutamyl-tRNA synthetase, another enzyme requiring the cognate tRNA to catalyze the isotope exchange (Lapointe & Söll, 1972; Kern et al., 1979). Three different approaches allowing predictions upon the existence of a one-step or a two-step glutamylation pathway have been used. First, we compared the efficiencies of $tRNA^{Glu}$ and Glu-$tRNA^{Glu}$ plus AMP on the [^{32}P]PP_i–ATP exchange. In the absence of formation of any intermediate in the overall aminoacylation process, one may expect that (1) the isotope exchange depends on the $tRNA^{Glu}$ concentration as well as on the concentrations of the end products Glu-$tRNA^{Glu}$ and AMP and (2) this exchange occurs faster after rather than before the establishment of the equilibrium of the aminoacylation reaction. On the other hand, if a stepwise aminoacylation process occurs, such as the adenylate pathway, the isotope exchange occurs at the equilibrium of the amino acid activation step and does not involve Glu-$tRNA^{Glu}$ and AMP. Second, we determined the rates of isotope exchange and of tRNA charging and its reversal (the Glu-$tRNA^{Glu}$ deacylation being AMP and PP_i dependent). Comparison of these rate constants makes it possible to show whether the tRNA charging step and its reversal occur sufficiently fast to be reasonably involved in the isotope exchange or not. Third, we investigated the involvement of an intermediate appearing in the presence of glutamyl-tRNA synthetase, Glu-$tRNA^{Glu}$, and AMP (but in the absence of PP_i) in the overall reverse of the aminoacylation reaction. This intermediate could be isolated under various conditions and showed properties similar to those of enzyme·adenylate complexes isolated for other synthetases.

Materials and Methods

Materials. Glutamyl-tRNA synthetase was purified, and the protein concentrations were determined as described previously (Kern et al., 1979). Unfractionated tRNA from *E. coli* B (acceptor capacity of glutamate = 4%) was purchased from Schwarz/Mann; most experiments were effected with this tRNA. Experiments establishing various stoichiometries, the rate-determining steps of the tRNA charging and its reversal, and the AMP-dependent deacylation reactions were effected with 90% purified $tRNA_2^{Glu}$ (Boehringer) or [^{14}C]Glu-$tRNA^{Glu}$. L-Glutamate, Hepes, ATP, AMP, and inorganic pyrophosphatase (one unit catalyzes the formation of 1 μmol of P_i at 25° C and pH 7.2) were from Sigma, and phosphodiesterase was from Worthington. $NaPP_i$ was a Fisher product; Sephadex G-75 (superfine) was from Pharmacia; [^{3}H]- and [^{14}C]-L-glutamate (respectively 25 μCi/mmol and 250 mCi/mmol), [^{32}P]PP_i, [γ-^{32}P]ATP, and Omnifluor were from New England Nuclear. ATP and AMP were extensively purified by chromatography on thin-layer plates of silica gel (60 F 254 from Merck) with an ethanol/water/ammonia (6:2:1 v/v/v) solvent.

[^{32}P]PP_i-ATP Exchange Kinetics. The reaction mixtures contained 200 mM Na–Hepes, pH 6.2, or 100 mM Na–Hepes, pH 7.4 or 8.6, 2 mM ATP, 16 mM $MgCl_2$, 1 mM L-glutamate, 1 mM Na[^{32}P]PP_i (2000 cpm/nmol), 1–6 μg of enzyme per mL (as indicated), and unless otherwise indicated 4.8 μM $tRNA^{Glu}$ or Glu-$tRNA^{Glu}$. After various incubation times at 37 °C, the [^{32}P]ATP synthesized was determined as described previously (Kern et al., 1979).

Aminoacylation Kinetics. The reaction mixtures contained 200 mM Na–Hepes, pH 6.2, or 100 mM Na–Hepes, pH 7.4 or 8.6, 2 mM ATP, 16 mM $MgCl_2$, 0.4 mM [^{14}C]L-glutamate (51 000 cpm/nmol), 4.8 μM $tRNA^{Glu}$, and 0.4 μg of enzyme per mL. After various incubation times at 37 °C (unless otherwise indicated), the [^{14}C]Glu-$tRNA^{Glu}$ synthesized was determined as described previously (Kern et al., 1979).

For large-scale preparations, $tRNA^{Glu}$ was charged under the conditions described above with either unlabeled or [^{14}C]glutamate (65 000 cpm/nmol), and the Glu-$tRNA^{Glu}$ was then isolated on a short DEAE-cellulose column at pH 4.5 (Yang & Novelli, 1968). The extent of aminoacylation was 95–100%.

AMP- and PP_i-Dependent Glu-$tRNA^{Glu}$ Deacylation Kinetics. The reaction mixtures contained 200 mM Na–Hepes, pH 6.2, or 100 mM Na–Hepes, pH 7.2 or 8.6, 10 mM AMP, 1 mM $NaPP_i$, 16 mM $MgCl_2$, 4.8 μM [^{14}C]Glu-$tRNA^{Glu}$ (65 000 cpm/nmol), and 6 μg of enzyme per mL. The crude results of deacylation were corrected by taking into account the chemical and enzymatic (AMP- and PP_i-independent) deacylations determined in the absence of either enzyme or AMP plus PP_i.

Parallel Kinetics of Aminoacylation or of Glu-$tRNA^{Glu}$ Deacylation (AMP and PP_i Dependent) and of [^{32}P]PP_i–ATP Exchange. The reaction mixtures were those described for the aminoacylation except that 7.5 mM L-glutamate (either unlabeled or ^{3}H-labeled: 15 000 cpm/nmol for measurements of aminoacylation), 1 mM $NaPP_i$ (either unlabeled or ^{32}P labeled: 2700 cpm/nmol for measurements of isotope exchange) and 1.8 μg of enzyme per mL were present. The parallel kinetics of the reversal of tRNA charging and isotope exchange were effected in the presence of Glu-$tRNA^{Glu}$ (either ^{14}C-labeled or unlabeled for measurements of isotope exchange) and 1 mM AMP.

AMP-Dependent and PP_i-Independent Glu-$tRNA^{Glu}$ Deacylation. The mixtures containing the various reactants at the indicated concentrations, except one (enzyme or AMP), were preincubated at 0 °C for 15 min in the presence of 3 units of inorganic pyrophosphatase per mL in order to destroy any contaminating PP_i. The omitted reactant was incubated separately in the presence of inorganic pyrophosphatase and the reaction started by mixing all reactants.

Preparation of $tRNA^{Glu}$-CCA. The –CCA end of $tRNA^{Glu}$ was removed with phosphodiesterase according to the procedure described by Zubay & Takanami (1964). After precipitation and dialysis, it was verified that the acceptor capacity of $tRNA^{Glu}$ was completely lost but could be restored in the presence of ATP, CTP, and trinucleotidyl-tRNA transferase.

Periodate Oxidation of $tRNA^{Glu}$. $tRNA^{Glu}$ was treated under the conditions described by Giegé et al. (1974) except that only 1 mM sodium metaperiodate was present.

Isolation of the Glutamyl-tRNA Synthetase·Glutamyl-adenylate Complex by Gel Filtration. The 200-μL reaction mixture containing the various reactants at the indicated concentrations was filtered through a Sephadex G-75 column (0.5 × 30 cm) equilibrated with 200 mM Na–Hepes, pH 6.2, 5 mM Na_2EDTA, and 5 mM 2-mercaptoethanol at 4 °C. The flow rate was 5 mL/h, and 135-μL fractions (except those containing the protein, which varied between 110 and 125 μL) were collected. The total [^{3}H]glutamate (as glutamate bound

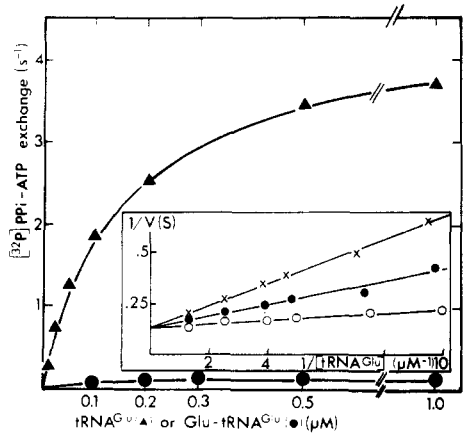

FIGURE 1: Influence of $tRNA^{Glu}$ and of Glu-$tRNA^{Glu}$ on the [^{32}P]-PP_i–ATP exchange. The reactions were conducted at pH 6.2 as described under Materials and Methods. Since in this reaction Glu-$tRNA^{Glu}$ acts as a competitive inhibitor with respect to $tRNA^{Glu}$ [$K_m(tRNA^{Glu})/K_i(\text{Glu-}tRNA^{Glu}) = 1$, see the insert], the contaminating $tRNA^{Glu}$ concentration in the Glu-$tRNA^{Glu}$ preparation which would be responsible for the stimulation of the isotope exchange is defined by $[tRNA^{Glu}] = v(K_m + [\text{Glu-}tRNA^{Glu}])/V - v$, where v and V correspond respectively to the isotope-exchange rates in the presence of a limiting $tRNA^{Glu}$ concentration (when Glu-$tRNA^{Glu}$ is tested) and in the presence of a saturating $tRNA^{Glu}$ concentration; k_m ($tRNA^{Glu}$) = 0.1 μM (see the insert). Thus the $tRNA^{Glu}$ contamination in the Glu-$tRNA^{Glu}$ preparation which would be responsible for the isotope exchange observed corresponds to 4%. This value is easily compatible with the extent of charging of $tRNA^{Glu}$ (see Materials and Methods). The insert shows the double reciprocal plots $1/v = f(1/[tRNA^{Glu}])$ in the absence of Glu-$tRNA^{Glu}$ (○) or in the presence of 0.1 (●) or 0.3 μM (×) Glu-$tRNA^{Glu}$.

to enzyme and as Glu-$tRNA^{Glu}$) was determined by counting aliquots of the eluted fractions in Bray's solution, and the [^{3}H]Glu-$tRNA^{Glu}$ was determined after trichloroacetic acid precipitation of aliquots. The [^{3}H]glutamate bound to the enzyme was obtained by the difference between both countings (which were previously corrected for the same specific activity of the ^{3}H labeling in both determinations). When present, [^{32}P]AMP was determined by counting aliquots of the fractions in Bray's solution.

Results

First Approach: Comparison of the Influence of $tRNA^{Glu}$ and of Glu-$tRNA^{Glu}$ Plus AMP on the [^{32}P]PP_i–ATP Exchange. Figure 1 shows that $tRNA^{Glu}$ stimulated the isotope exchange much more efficiently than Glu-$tRNA^{Glu}$ did. The slow rate of [^{32}P]ATP synthesis observed in the latter case was attributed to the presence of $tRNA^{Glu}$ contaminating the Glu-$tRNA^{Glu}$ preparation (see the legend to Figure 1). In addition, Glu-$tRNA^{Glu}$ acted as a competitive inhibitor respective to $tRNA^{Glu}$ in this reaction (Figure 1, insert). These observations agree with other experiments showing that tRNAs unable to accept the amino acid cannot promote the isotope exchange catalyzed by the glutamyl-, glutaminyl-, and arginyl-tRNA synthetases of various organisms (Lapointe & Söll, 1972; Kern et al., 1979; Folk, 1971; Mehler & Mitra, 1967; Parfait & Grosjean, 1972; Nazario & Evans, 1974; Gangloff et al., 1976).

The effect of Glu-$tRNA^{Glu}$ on the isotope exchange was further studied in the presence of AMP. Table I shows that the exchange occurred much faster in the presence of $tRNA^{Glu}$ (experiment 1) than in the presence of a stoichiometric concentration of Glu-$tRNA^{Glu}$ and AMP, which would have resulted from the aminoacylation of $tRNA^{Glu}$ (experiment 2). In the presence of Glu-$tRNA^{Glu}$, an excess of AMP (1 mM) significantly stimulated the rate of the isotope exchange (experiment 3), which, however, remained lower than the rate observed in the presence of $tRNA^{Glu}$ (experiment 1). Finally, the slow rate observed in the presence of Glu-$tRNA^{Glu}$ and in the absence of added AMP (experiment 4) was due to contaminating $tRNA^{Glu}$ (see the legend to Figure 1).

Table I: Comparison of the Influence of $tRNA^{Glu}$ and of Glu-$tRNA^{Glu}$ Plus AMP on the Rate of [^{32}P]PP_i-ATP Exchange[a]

expt no.	$tRNA^{Glu}$ (μM)	addition of Glu-$tRNA^{Glu}$ (μM)	AMP (μM)	isotope exchange initial rate (s^{-1})[b]
1	2	0	0	2.2
2	0	2	2	0.17
3	0	2	1000	1.0
4	0	2	0	0.14

[a] The reaction mixture contained 100 mM Na-Hepes, pH 7.4, 2 mM ATP, 1 mM $NaPP_i$ (2300 cpm/nmol), 16 mM $MgCl_2$, 1 mM L-glutamate, and 18 nM enzyme. [b] The rates are expressed as nmol of [^{32}P]ATP formed per nmol of enzyme per second.

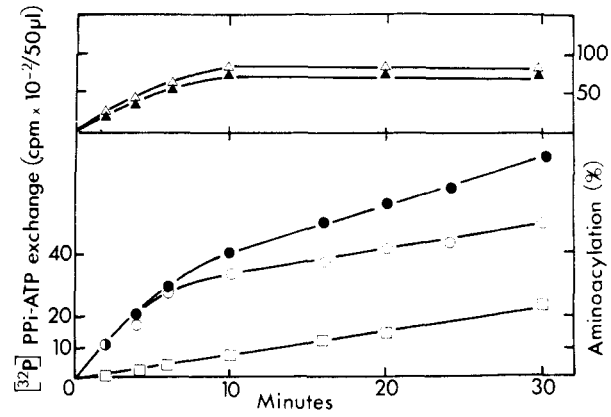

FIGURE 2: Parallel kinetics of aminoacylation (△,▲) and of [^{32}P]PP_i–ATP exchange (○,●). The reactions were effected at pH 7.4, as described under Materials and Methods, in the presence of 4.8 μM $tRNA^{Glu}$ without AMP (△,○) or with 1 mM AMP (●,▲). In one experiment (□) the isotope exchange was followed in the presence of ligand concentrations corresponding to those present at the chemical equilibrium: 4.15 μM Glu-$tRNA^{Glu}$, 0.65 μM $tRNA^{Glu}$, 0.65 μM AMP, and 1 mM PP_i.

Several control experiments were performed: (1) one ATP was consumed per Glu-$tRNA^{Glu}$ synthesized (result not shown), indicating that the enzyme had no ATPase activity; (2) no significant chemical ATP hydrolysis occurred under the experimental conditions (half-life greater than 3 h); (3) the additional AMP produced as a consequence of the turnover of the Glu-$tRNA^{Glu}$ during the incubation time was not significant compared with that produced during the first aminoacylation of $tRNA^{Glu}$; indeed, the rate constants of the aminoacylation (k_a), the chemical deacylation (k_c), and the enzymatic deacylation (k_b) are respectively $k_a = 1.5\ s^{-1}$, $k_c = 0.31 \times 10^{-3}\ s^{-1}$, and $k_b < 0.06 \times 10^{-3}\ s^{-1}$. These controls showed that no secondary ATPase activity occurred which would have given an amount of AMP higher than expected from the aminoacylation reaction alone.

These results show that $tRNA^{Glu}$ stimulates the isotope exchange better than Glu-$tRNA^{Glu}$ plus AMP. A consequence of this is the particular kinetic behavior of the isotope exchange during the aminoacylation reaction. Indeed, these kinetics were found to be biphasic, the decrease in the rate occurring at the beginning of the aminoacylation plateau. These kinetics were studied at pH 6.2, 7.4, and 8.6, which were found to be respectively minimal, intermediate, and optimal pH values for the aminoacylation reaction and vice versa for the isotope

Table II: Rates of [^{32}P]PP_i–ATP Exchange and of tRNA Charging in the Presence of Initially Uncharged $tRNA^{Glu}$ [a]

pH	rate of aminoacylation (s^{-1})[b]	rate of [^{32}P]PP_i–ATP exchange (s^{-1})[b]			
		fast phase		slow phase	
		−AMP	+AMP	−AMP	+AMP
6.2	0.12	3.69	3.69	c	c
7.4	1.50	2.2	2.2	0.30	0.62
8.6	3.73	1.15	1.15	0.03	0.31

[a] The conditions are as described under Materials and Methods. [b] The rates are expressed as nanomoles of end product formed per nanomole of enzyme per second. [c] Not determined.

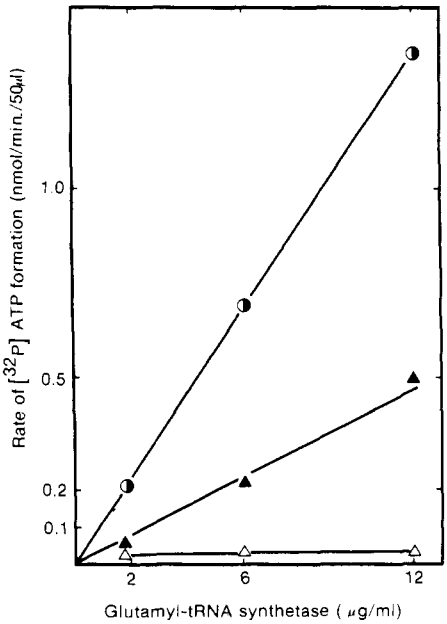

FIGURE 3: Evolution of the two phases of the [^{32}P]PP_i–ATP exchange as a function of the concentration of glutamyl-tRNA synthetase. The experiments were conducted at pH 7.4 under the conditions described under Materials and Methods: rate of the first (fast) phase in the absence or in the presence of 1 mM AMP (◑); rate of the second (slow) phase in the absence (△) or in the presence of 1 mM AMP (▲).

exchange. Figure 2 shows the kinetics at pH 7.4. The addition of 1 mM AMP did not modify the rate of the first (fast) phase but increased that of the second (slow) phase. In parallel, the aminoacylation extents of tRNA at the plateaus were found to be decreased in the presence of 1 mM AMP. The values of the rate constants of aminoacylation and of the isotope exchange are given in Table II.

The isotope exchange was studied as a function of the [$tRNA^{Glu}$]/[Glu-$tRNA^{Glu}$ + AMP] ratio at the aminoacylation plateau. Applying the tRNA aminoacylation plateau theory of Bonnet & Ebel (1972), we modulated the value of this ratio at the aminoacylation plateau by varying the enzyme concentration. Figure 3 shows that (1) the initial exchange rate was proportional to the enzyme concentration, (2) this proportionality disappeared at the aminoacylation plateau, and (3) it appeared now at the plateau in the presence of 1 mM AMP. In order to discriminate accurately between the stimulation of isotope exchange at the aminoacylation plateau either by $tRNA^{Glu}$ or by Glu-$tRNA^{Glu}$ plus AMP, we determined the concentrations of these reactants at the plateau by two independent methods (see Table III and the legend). In the absence of added AMP a very good agreement was shown between (1) the $tRNA^{Glu}$ concentrations, determined by assuming that the aminoacylation plateau resulted from an equilibrium between the aminoacylation and the deacylation

Table III: Effect of Concentration of Glutamyl-tRNA Synthetase on Extent of Charging of $tRNA^{Glu}$ at the Aminoacylation Plateau and Relation between This Extent and the Stimulation of Isotope Exchange[a]

Glu-tRNA synthetase (µM)	[$tRNA^{Glu}$] (µM) at the plateaus determined according to			
	(A) −AMP	(A) +AMP	(B) −AMP	(C) +AMP
0.032	0.67	1.40	0.65	1.13
0.105	0.23	1.55	0.23	0.71
0.210	0.13	1.71	0.12	0.60

[a] The values were extracted from the experiments reported in Figure 3. The concentrations of $tRNA^{Glu}$ at the aminoacylation plateaus were determined by the following approaches. (A) Let us assume that the [^{32}P]PP_i–ATP isotope exchange at the aminoacylation plateau is promoted by $tRNA^{Glu}$ and inhibited competitively by Glu-$tRNA^{Glu}$. Since $K_m(tRNA^{Glu})/K_i(\text{Glu-}tRNA^{Glu}) = 1$ (see above), the rate of isotope exchange (v) in the presence of a given concentration of [$tRNA^{Glu}$] and [Glu-$tRNA^{Glu}$] is related to the maximal rate of this exchange (V) when $tRNA^{Glu}$ is saturating by $v = V[tRNA^{Glu}]/K_m[tRNA^{Glu}] + [\text{Glu-}tRNA^{Glu}]$. (B) Let us assume that the aminoacylation plateaus result from an equilibrium between the aminoacylation reaction and various deacylation reactions. Thus, the equation established by Bonnet & Ebel (1972) gives the real extent of charged tRNA: $[tRNA^{Glu}]_0/[\text{Glu-}tRNA^{Glu}] = [k_b(K_m + [tRNA^{Glu}]_0)/k_a](1/[E]) + (k_c + k_d)/k_a + 1$. $[tRNA^{Glu}]_0$ and [Glu-$tRNA^{Glu}$] correspond respectively to the concentrations of $tRNA^{Glu}$ at $t = 0$ and the aminoacylated tRNA at the plateau; $K_m = 0.15$ µM at pH 7.4; k_a, k_b, k_c, and k_d correspond respectively to the rate constants of aminoacylation, of chemical and enzymatic deacylations either AMP and PP_i independent or dependent. Under the experimental conditions used, the following rate constant values were determined: $k_a = 0.31\ s^{-1}$, $k_b = 0.00031\ s^{-1}$, k_c was found lower than $0.0006\ s^{-1}$, and $k_d = 0.0006\ s^{-1}$. (C) In the presence of 1 mM AMP, the extent of tRNA charging was determined in the acidic precipitate of aliquots of the incubation mixture.

reactions, and (2) those which would take into account the stimulation of the isotope exchange. These results (1) confirm what was shown by initial velocity experiments, i.e., that under standard aminoacylation conditions $tRNA^{Glu}$ acts as an activator and Glu-$tRNA^{Glu}$ (even in the presence of a stoichiometric AMP concentration) as an inhibitor of the isotope exchange, and (2) demonstrate that the decrease of the isotope-exchange rate during tRNA aminoacylation results from the conversion of $tRNA^{Glu}$ to Glu-$tRNA^{Glu}$. However, in the presence of 1 mM AMP (a concentration much higher than that generally produced during the aminoacylation), the isotope exchange occurred faster than expected (Table III). Thus, under these conditions (which do not correspond to standard aminoacylation conditions), Glu-$tRNA^{Glu}$ acted not only as an inhibitor but also took part with AMP in the incorporation of [^{32}P]PP_i into ATP via the reverse of the aminoacylation reaction. These results indicate that Glu-$tRNA^{Glu}$ and AMP are not obligatory substrates of the isotope exchange.

Of interest to mention is the behavior of the isotope-exchange kinetics during the AMP- and PP_i-dependent glutamyl-tRNA synthetase catalyzed Glu-$tRNA^{Glu}$ deacylation reaction. In the absence of added glutamate and ATP, the incorporation of [^{32}P]PP_i into ATP was in a stoichiometric ratio of 1:1 with the Glu-$tRNA^{Glu}$ deacylated (results not shown). However, in the presence of high ATP and glutamate concentrations (~1 mM), Glu-$tRNA^{Glu}$ and AMP could promote the isotope exchange. Under these conditions, the kinetics of the exchange were also biphasic, the rate decrease corresponding to the establishment of the deacylation plateau (Figure 4). Table IV compares the rates of deacylation of Glu-$tRNA^{Glu}$ (AMP and PP_i dependent) with the isotope-

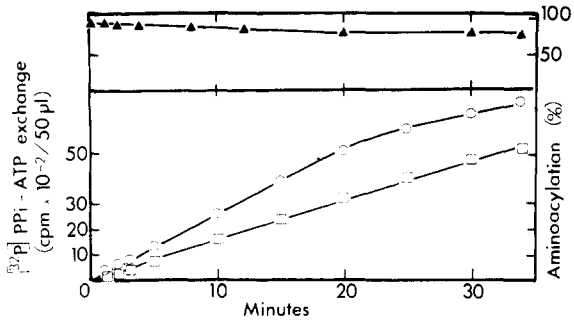

FIGURE 4: Parallel kinetics of the AMP- and PP_i-dependent Glu-$tRNA^{Glu}$ deacylation (▲) and of the [^{32}P]PP_i–ATP exchange (○). The reactions were conducted at pH 7.4, as described under Materials and Methods, in the presence of 4.8 µM Glu-$tRNA^{Glu}$. In one experiment (□), the isotope exchange was followed in the presence of ligand concentrations corresponding to those present at the chemical equilibrium: 3.7 µM Glu-$tRNA^{Glu}$, 1.1 µM $tRNA^{Glu}$, 1 mM AMP, and 1 mM PP_i.

Table IV: Rates of [^{32}P]PP_i–ATP Exchange Promoted by Glu-$tRNA^{Glu}$ Plus AMP and of AMP- and PP_i-Dependent Glu-$tRNA^{Glu}$ Deacylation[a]

pH	rate of deacylation (s^{-1})[b]	rate of [^{32}P]PP_i–ATP exchange (s^{-1})[b] fast phase	rate of [^{32}P]PP_i–ATP exchange (s^{-1})[b] slow phase	exchange / deacylation
6.2	0.046	2.1	[c]	46
7.4	0.24	1.0	0.63	3.7
8.6	0.37	0.62	0.31	1.67

[a] The conditions are as described under Materials and Methods. [b] The rates are expressed as nanomoles of end product formed per nanomole of enzyme per second. [c] Not determined.

exchange rates under these conditions at pH 6.2, 7.4, and 8.6. It appears that the first (fast) rate of the exchange increased with the pH decrease. Since the affinity of $tRNA^{Glu}$ for glutamyl-tRNA synthetase varied in the same manner with pH as the rate of the fast phase of isotope exchange (results not shown), these results suggest that after the deacylation step, $tRNA^{Glu}$ promotes several catalytic cycles of isotope exchange before its dissociation from the enzyme·$tRNA^{Glu}$·ATP·Glu end-product complex.

Finally, the isotope exchange occurred at the same rate either at the aminoacylation plateau (when the reaction is initiated by $tRNA^{Glu}$) or at the deacylation plateau (when the reaction is initiated by Glu-$tRNA^{Glu}$ and AMP), or when $tRNA^{Glu}$, Glu-$tRNA^{Glu}$, and AMP were directly mixed at concentrations known to be present at these plateaus (Figures 2 and 4).

Second Approach: Comparison of the Rate of [^{32}P]PP_i–ATP Isotope Exchange and the Rates of Aminoacylation and Its Reversal. Tables II and IV compare the rate constants of these reactions at three pH values. It appears (1) that in the presence of $tRNA^{Glu}$ the isotope exchange occurred from pH 6.2 to 7.4 significantly faster than the aminoacylation of $tRNA^{Glu}$ and (2) that in the presence of Glu-$tRNA^{Glu}$ plus AMP, the isotope exchange occurred from pH 6.2 to 8.6 faster than the reversal of the tRNA charging. However, these results can correctly be interpreted in the context of a one-step or a two-step aminoacylation pathway only after determination of the rate-limiting steps of the overall glutamylation process and its reversal.

When the initial rate of Glu-$tRNA^{Glu}$ synthesis was measured under experimental conditions where several catalytic cycles of the enzyme could be followed, linear curves which extrapolated back through the origin of the graph were obtained at pH 6.2 and 7.4 (Figure 5A). The same approach applied to the AMP- and PP_i-dependent Glu-$tRNA^{Glu}$ deacylation reaction showed no decrease in the rate after the first catalytic cycle at pH 6.2 and 7.4 (Figure 5B). These results indicate that no end-product dissociation is rate determining at the steady state of the overall aminoacylation reaction and its reversal. Thus the steady-state rates are probably a measure of the catalysis of both reactions. Since the absolute rates of $tRNA^{Glu}$ charging and of its reversal are significantly slower than the rate of isotope exchange, it seems a priori that the two former reactions are not involved in the latter one.

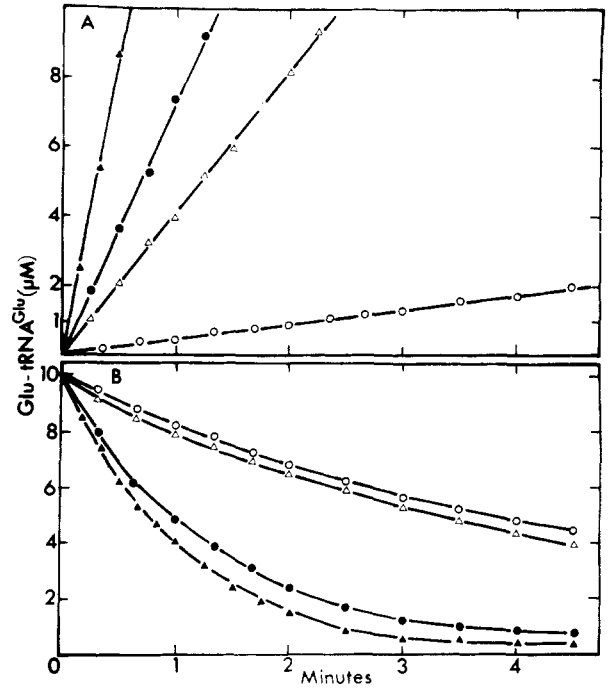

FIGURE 5: Rate-determining steps of the overall aminoacylation reaction (A) and its reversal (B). (A) The incubation mixtures contained 200 mM Na–Hepes, pH 6.2 (○,●), or 100 mM Na–Hepes, pH 7.4 (△,▲), 2 mM ATP, 16 mM $MgCl_2$, 50 µM $tRNA^{Glu}$, 0.5 mM [^{14}C]-L-glutamate (32 400 cpm/nmol), and 1 µM enzyme. The reactions were conducted either at 0 °C (○,△), at 20 °C (▲), or at 37 °C (●). (B) The incubation mixtures contained 200 mM Na–Hepes, pH 6.2 (○,●), or 100 mM Na–Hepes, pH 7.4 (△,▲), 10 mM AMP, 1 mM $NaPP_i$, 16 mM $MgCl_2$, 10 µM [^{14}C]Glu-$tRNA^{Glu}$ (65 000 cpm/nmol) and 2 µM enzyme. The reactions were conducted at 0 °C (△), at 20 °C (○,▲), or at 37 °C (●).

Third Approach: Detection of the Enzyme·Aminoacyl-adenylate Intermediate. (1) Kinetic Detection. In the presence of AMP, and in the absence of PP_i, the glutamyl-tRNA synthetase catalyzed the deacylation of a fraction of the Glu-$tRNA^{Glu}$ present in the incubation mixture before a stable deacylation plateau was established at either pH 6.2 (Figure 6A) or 7.2 (Figure 6B). In the presence of an excess of PP_i (2 mM) the Glu-$tRNA^{Glu}$ was totally deacylated (Figure 6). When a small amount of PP_i (20 µM) was added to the reaction mixture, different deacylation kinetics were obtained, depending on whether the mixture was or was not preincubated in the presence of inorganic pyrophosphatase (Figure 6B). In the presence of pyrophosphatase the deacylation reaction occurred as in the absence of added PP_i. However, in the absence of pyrophosphatase, no deacylation plateau was obtained, and a biphasic deacylation kinetic reaction was observed. The initial rate of deacylation was the same whether the reaction was done in the absence or in the presence of PP_i (20 µM or 2 mM) (Figure 6). These facts mean that the pyrophosphatase was active and that glutamyl-tRNA synthetase catalyzed an AMP-dependent and PP_i-independent deacylation.

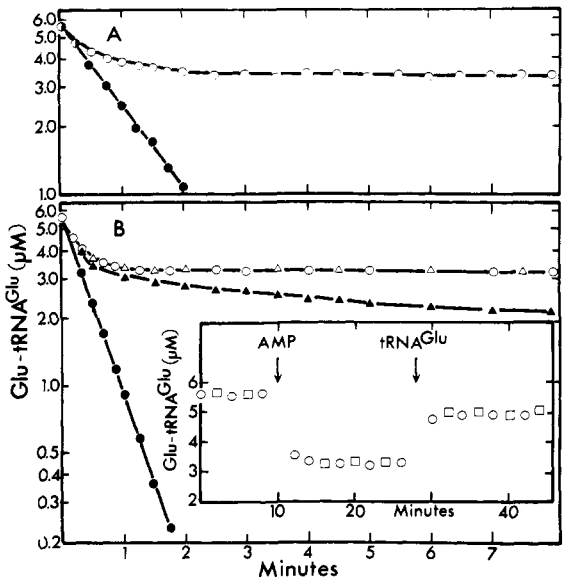

FIGURE 6: AMP- and PP_i-dependent and the AMP-dependent and PP_i-independent glutamyl-tRNA synthetase catalyzed Glu-tRNA^{Glu} deacylations. The incubation mixtures contained 200 mM Na-Hepes, pH 6.2 (A), or 100 mM Na-Hepes, pH 7.4 (B), 16 mM $MgCl_2$, 5 mM AMP, 5.6 μM [^{14}C]Glu-tRNA^{Glu}, 0.4 μM tRNA^{Glu}, and 15.2 μM glutamyl-tRNA synthetase. The reactions were effected at 20 °C (A) or at 0 °C (B) in the absence of PP_i (○) or in the presence of 20 μM PP_i (△,▲) or 2 mM PP_i (●), without preincubation (●,▲) or after preincubation in the presence of 3.0 units of inorganic pyrophosphatase per mL (○,△). The insert in (B) shows the equilibrium of the AMP-dependent and PP_i-independent Glu-tRNA^{Glu} deacylation reaction. The various reactants (except AMP) in concentrations as indicated for (○) in (B) were preincubated in the presence of inorganic pyrophosphatase; the reaction was started by addition of 5 mM AMP as indicated by the first arrow. The second arrow indicates the addition of 10 μM tRNA^{Glu}. A control experiment (□) was effected under the same conditions except that 10 μM glutamate was present.

The addition of free tRNA^{Glu} at the deacylation plateau resulted in a fast synthesis of additional [^{14}C]Glu-tRNA^{Glu} (Figure 6B, insert) which was promoted neither by contaminating ATP in the incubation mixture nor by free [^{14}C]-glutamate which could be produced by the hydrolysis of [^{14}C]Glu-tRNA^{Glu}. Indeed, if this had occurred, the isotopic dilution of the [^{14}C]glutamate should have decreased the apparent amount of [^{14}C]Glu-tRNA^{Glu} synthesized after addition of tRNA^{Glu} at the deacylation plateau. The insert in Figure 6B shows that the addition in the incubation mixture of an amount of unlabeled glutamate, even significantly higher than that which could be produced by a complete hydrolysis of the initially present Glu-tRNA^{Glu}, did not affect the amount of the [^{14}C]Glu-tRNA^{Glu} synthesized. Thus, the glutamyl-tRNA synthetase is able to catalyze the synthesis of Glu-tRNA^{Glu} via a reaction requiring neither free ATP nor glutamate as a substrate, suggesting the existence of the equilibrated reaction:

$$E + \text{Glu-tRNA}^{Glu} + AMP \rightleftharpoons E{\cdot}AMP{\sim}Glu + \text{tRNA}^{Glu} \quad (3)$$

The equilibrium of this reaction was found to depend upon the concentrations of tRNA^{Glu}, Glu-tRNA^{Glu}, AMP, and enzyme (results not shown). By analogy with the two-step model of aminoacylation proposed for other synthetases, we propose the involvement of an enzyme·aminoacyladenylate complex in this reaction.

(2) Isolation of the Intermediate. The intermediate was generated via the reversal of the previously established reaction (eq 3), and its isolation was then attempted by gel filtration under various conditions. When the reaction mixture containing glutamyl-tRNA synthetase, [^{3}H]Glu-tRNA^{Glu}, [^{32}P]AMP, and inorganic pyrophosphatase was filtered through a G-75 Sephadex column, the [^{3}H]Glu-tRNA^{Glu} initially added was quantitatively recovered in the macromolecular fraction (containing the enzyme, tRNA^{Glu}, and Glu-tRNA^{Glu}), even when before chromatography of the mixture a part of the acid-precipitable [^{3}H]Glu-tRNA^{Glu} disappeared (cf. preceding section). Furthermore, no [^{32}P]AMP was found in the macromolecular fraction (Table V, experiment 1). When the Sephadex column was equilibrated with the same AMP concentration as that present in the mixture of reactants, the [^{3}H]Glu-tRNA^{Glu} recovered in the macromolecular fraction was that initially present in the reaction mixture; additional [^{3}H]glutamate was found present in this macromolecular fraction (experiment 2A). Finally, the addition of tRNA^{Glu} to the eluted macromolecular fraction (experiment 2B) gave rise to the synthesis of [^{3}H]Glu-tRNA^{Glu}, indicating that the labeled glutamate that had disappeared from [^{3}H]-Glu-tRNA^{Glu} remained linked to the enzyme via a high-energy bond able to transfer further the amino acid to tRNA in the absence of ATP.

The introduction of a large excess of tRNA^{Glu}-CCA in the reactant mixture containing [^{3}H]Glu-tRNA^{Glu} and [^{32}P]AMP after the establishment of the equilibrium of reaction 3 resulted in a stabilization of the various components present. Indeed, the free enzyme, as well as the enzyme·adenylate complex, was mainly saturated with tRNA^{Glu}-CCA unable to promote the reactions in both ways. After filtration of this mixture on a G-75 Sephadex column not even equilibrated with AMP, the part of the labeled glutamate which disappeared from [^{3}H]-Glu-tRNA^{Glu} (consecutively to the AMP-promoted Glu-tRNA^{Glu} deacylation reaction) remained associated with the macromolecular fraction eluted; in addition, an amount of [^{32}P]AMP which corresponded to that of deacylated Glu-

Table V: Detection of Glutamyl-tRNA Synthetase·Glutamyladenylate Complex by Gel Filtration

	macromolecular fraction			micromolecular fraction	
expt[a]	Glu-tRNA^{Glu} (pmol)	glutamate (pmol)	AMP (pmol)	glutamate (pmol)	AMP (nmol)
1	1108	0	0	0	≃1000
2A	664	442	*b*	0	*b*
2B	950	156			
3	658	444	436	0	≃1000
4	648	21	19	235	≃1000

[a] The 200-μL reaction mixtures contained 200 mM Na-Hepes, pH 6.2, 1 mM $MgCl_2$, 5.6 μM [^{3}H]Glu-tRNA^{Glu}, 0.4 μM tRNA tRNA^{Glu}, 5 mM AMP [either ^{32}P-labeled (6.1 cpm/pmol) in experiments 1, 3, and 4 or not labeled in experiment 2A], and 3 units of inorganic pyrophosphatase per mL. The addition of 15.2 μM glutamyl-tRNA synthetase resulted in a decrease of the concentration of [^{3}H]Glu-tRNA^{Glu} to 3.35 μM. This mixture was then filtered through a Sephadex G-75 column either in the absence of AMP (experiments 1, 3, and 4) or under equilibration with 5 mM AMP (experiment 2A), and the [^{3}H]Glu-tRNA^{Glu}, [^{3}H]glutamate, and [^{32}P]AMP (when present) contents of the macromolecular and micromolecular fractions were determined as described under Materials and Methods. In experiment 2B, 10 μM tRNA^{Glu} was added to the macromolecular fraction eluted in experiment 2A. In experiment 3, 200 μM tRNA^{Glu}-CCA was added to the reactant mixture before gel filtration. We verified that K_m(tRNA^{Glu})/K_i(tRNA^{Glu}-CCA) in the isotope exchange ≃ 1. In experiment 4, 200 μM periodate-oxidized tRNA^{Glu} was added to the reactant mixture before gel filtration [K_m·(tRNA^{Glu})/K_i(periodate-oxidized tRNA^{Glu}) = 1]. The amount of glutamate present in the micromolecular fraction was determined in a parallel experiment effected in the presence of unlabeled AMP. [b] Not determined.

$tRNA^{Glu}$ was found present in this fraction (experiment 3 in Table V).

Periodate-oxidized $tRNA^{Glu}$ which is another $tRNA^{Glu}$ species unable to promote the reactions of eq 3 was found unable to replace $tRNA^{Glu}$-CCA in this approach. Indeed, the introduction of this modified $tRNA^{Glu}$ in the mixture of reactants after establishment of the equilibrium resulted in the elution of the [^{3}H]glutamate which initially disappeared from [^{3}H]Glu-$tRNA^{Glu}$ mainly in the micromolecular fraction. No significant amounts of [^{3}H]glutamate and [^{32}P]AMP were found in the macromolecular fraction, but radioactivity was present in the intermediate fractions (experiment 4 in Table V). These results probably indicate a high instabilty of one of the intermediates of the equilibrated reaction (3) when periodate-oxidized $tRNA^{Glu}$ is present. In this context it is interesting to mention that the isolated adenylate intermediates of the yeast phenylalanyl-tRNA synthetase (Baltzinger & Remy, 1977) and of the yeast valyl-tRNA synthetase (Kern, unpublished experiments) were found very unstable in the presence of their cognate periodate-oxidized tRNA. Our results suggest that periodate-oxidized $tRNA^{Glu}$ destabilizes the glutamyl-tRNA synthetase·glutamyladenylate complex.

Discussion

The experiments reported in this paper were designed to discriminate for glutamyl-tRNA synthetase between the concerted mechanism and the two-step mechanism generally proposed for the aminoacylation of tRNAs (Loftfield, 1972; Kisselev & Favorova, 1974; Söll & Schimmel, 1974; Kalousek & Konigsberg, 1975; Schimmel & Söll, 1979). Apart from different aminoacylation pathways, both mechanisms also predict different pathways for the [^{32}P]PP_i–ATP exchange and for the reversal of the tRNA charging. Indeed, in the two-step pathway (eq 1) the isotope exchange occurs independently of the tRNA charging and its reversal, and this last reaction is PP_i independent, whereas in the one-step pathway (eq 2) the isotope exchange obligatorily involves the reversal of the tRNA charging, and this last reaction is PP_i dependent.

Evidence for a Two-Step Mechanism for Glutamyl-tRNA Synthetase. The first experimental evidence which apparently favors the existence of a stepwise mechanism for $tRNA^{Glu}$ charging is that the rates of $tRNA^{Glu}$ charging and its reversal are both slower than the isotope exchange under various conditions, suggesting that neither the tRNA charging step nor its reversal are involved in the isotope exchange. However, as discussed by Fersht et al. (1978), such results are also compatible with a concerted mechanism involving just a slow conformational change of the enzyme. This change could either succeed the catalytic step

$$\begin{aligned} &E + tRNA + \\ &\quad ATP + Glu \xrightarrow{fast} E\cdot tRNA\cdot ATP\cdot Glu \\ &\xrightarrow{fast} E^*\cdot Glu\text{-}tRNA\cdot AMP\cdot PP_i \\ &\xrightarrow{slow} E\cdot Glu\text{-}tRNA\cdot AMP\cdot PP_i \\ &\xrightarrow{fast} E + Glu\text{-}tRNA + AMP + PP_i \end{aligned} \quad (4)$$

or precede it:

$$\begin{aligned} &E + tRNA + \\ &\quad ATP + Glu \xrightarrow{fast} E\cdot tRNA\cdot ATP\cdot Glu \\ &\xrightarrow{slow} E^*\cdot tRNA\cdot ATP\cdot Glu \\ &\xrightarrow{fast} E^*\cdot Glu\text{-}tRNA\cdot AMP\cdot PP_i \\ &\xrightarrow{fast} E\cdot Glu\text{-}tRNA\cdot AMP\cdot PP_i \\ &\xrightarrow{fast} E + Glu\text{-}tRNA + AMP + PP_i \end{aligned} \quad (5)$$

Such mechanisms allow a fast [^{32}P]PP_i–ATP exchange, the rates of the overall aminoacylation and its reversal being limited by the slow conformational change $E \leftrightarrow E^*$.

However, there are strong arguments against the existence of such pathways for glutamyl-tRNA synthetase. First, the $tRNA^{Glu}$ charging kinetics remain linear during and after the first turnover of the synthetase contrary to that expected from pathway 4 where the rate-determining conformational change $E^* \rightarrow E$ implies a decrease in the rate of aminoacylation after completion of the first turnover. Thus, the enzyme state change which could be rate determining for the $tRNA^{Glu}$ charging but not for the isotope exchange would obligatorily occur before the catalytic step; as a consequence pathway 4 can be excluded for glutamyl-tRNA synthetase. There are further arguments against pathway 5: no decrease in the rate of deacylation of Glu-$tRNA^{Glu}$ AMP and PP_i dependent occurs after completion of the first catalytic cycle of the enzyme, indicating that contrary to that expected from scheme 5 no rate-determining enzyme state change $E^* \rightarrow E$ succeeds the catalysis of the reversal of $tRNA^{Glu}$ charging. Nevertheless, this pathway cannot be definitively excluded, since it can be postulated that, independently of the way of the reaction, the first transconformation, $E \rightarrow E^*$, always occurs slower than the second one, $E^* \rightarrow E$.

Another, though highly unlikely, kinetic situation that could account for the rapid $tRNA^{Glu}$-dependent pyrophosphate exchange on the concerted scheme is that the exchange is a parallel side reaction that is dependent on both $tRNA^{Glu}$ and glutamate and is inhibited by Glu-$tRNA^{Glu}$. However, there is strong evidence that the reversal of $tRNA^{Glu}$ charging takes place in two chemical steps. As shown in Figure 6, glutamyl-tRNA synthetase catalyzes an AMP-dependent and PP_i-independent deacylation of Glu-$tRNA^{Glu}$; the initial rate of this reaction is the same as when PP_i is present, suggesting strongly that this partial step takes part in the overall reversal of $tRNA^{Glu}$ charging and constitutes the rate-determining step of this reaction. Thus, by analogy with the stepwise mechanism, this last reaction would be

$$\begin{aligned} E + Glu\text{-}tRNA + AMP &\xrightarrow{slow} E\cdot AMP\sim Glu + tRNA \\ + PP_i &\xrightarrow{fast} E + ATP + Glu \end{aligned} \quad (6)$$

Nature of the Intermediate Involved in the Glutamylation Process. The AMP-dependent and PP_i-independent deacylation of Glu-$tRNA^{Glu}$ gives rise to the synthesis of an enzyme complex to which AMP and glutamate remain associated in a 1:1 stoichiometry. This reaction was shown to be equilibrated according to eq 3; indeed, the decrease of the AMP concentration (when the mixture of reactants was filtered in the absence of equilibrating AMP along the column) or the increase of the $tRNA^{Glu}$ concentration in the reactants mixture both result in a displacement of the equilibrium toward the synthesis of Glu-$tRNA^{Glu}$. Thus glutamate is linked in this complex via a high-energy bond, and the properties of the intermediate agree well with those expected for the enzyme·adenylate complex.

The intermediate generated via the reversal of the transfer could be isolated after freezing the reactants present at the chemical equilibrium of this step by addition of a large excess of $tRNA^{Glu}$-CCA. Because of its strong competing effect with respect to $tRNA^{Glu}$ and Glu-$tRNA^{Glu}$, this modified $tRNA^{Glu}$ hindered further interactions of these ligands with any enzyme form and in particular with the enzyme·adenylate complex. As a consequence, the stabilized intermediate could be isolated by gel filtration of the reactant mixture in the absence of AMP, even after disruption of the equilibrium. The destabilizing

effect of periodate-oxidized $tRNA^{Glu}$ on the intermediate, which is a priori surprising, can be interpreted in two ways: (1) the oxidized and nonacceptor CCA end of $tRNA^{Glu}$ decreases the stability of the high-energy bond between glutamate and AMP either directly or indirectly by a steric effect (e.g., by modifying the surrounding of AMP~Glu); (2) if the transfer involves the synthesis of a highly labile acyl-enzyme intermediate as suggested for yeast phenylalanyl-tRNA synthetase (Rêmy & Ebel, 1976), and if periodate-oxidized $tRNA^{Glu}$ promotes the synthesis of this intermediate without being able to accept further the amino acid, the instability of AMP~Glu would reflect that of the acyl-enzyme intermediate.

[^{32}P]PP_i–ATP Exchange Catalyzed by Glutamyl-tRNA Synthetase. A stepwise mechanism for this synthetase implies an absolute requirement of free $tRNA^{Glu}$ (and not Glu-$tRNA^{Glu}$ as expected by the concerted mechanism) for the catalysis of the isotope exchange, since this substrate promotes the amino acid activation. We found actually that Glu-$tRNA^{Glu}$ is by itself unable to promote the isotope, exchange; however, when high concentrations of AMP were present, it could significantly stimulate this reaction. These kinetic behaviors are, however, only apparently contradictory. Indeed, when $tRNA^{Glu}$ is present, (1) Glu-$tRNA^{Glu}$ acts as a competitive inhibitor with respect to $tRNA^{Glu}$, (2) AMP does not affect the isotope exchange, and (3) the isotope exchange occurs significantly faster than in the presence of Glu-$tRNA^{Glu}$ plus AMP, indicating that $tRNA^{Glu}$ alone and not Glu-$tRNA^{Glu}$, either in the absence or in the presence of AMP, is directly involved in this reaction. Thus, in the presence of free $tRNA^{Glu}$ the isotope exchange is

$$E\cdot tRNA^{Glu}\cdot Glu\cdot ATP\cdot MgCl_2 \rightleftharpoons E\cdot tRNA^{Glu}\cdot AMP\sim Glu\cdot PP_i\cdot MgCl_2$$

In the presence of Glu-$tRNA^{Glu}$ and a high concentration of AMP (much higher than that usually generated during aminoacylation in the tRNA charging media), the isotope exchange results from a slow dissociation of $tRNA^{Glu}$ (generated by the AMP-dependent deacylation of Glu-$tRNA^{Glu}$) and a fast dissociation of ATP from the end-product complex. Under these conditions the isotope exchange is

$$E\cdot Glu\text{-}tRNA^{Glu}\cdot AMP\cdot PP_i\cdot MgCl_2 \xrightarrow{fast} E\cdot tRNA^{Glu}\cdot AMP\sim Glu\cdot PP_i\cdot MgCl_2 \xrightleftharpoons{fast} E\cdot tRNA^{Glu}\cdot ATP\cdot MgCl_2\cdot Glu \xrightarrow{slow} E + tRNA^{Glu} + ATP\cdot MgCl_2 + Glu$$

Finally, when the substrates of the forward and of the reverse reactions are present at high concentrations, allowing a fast turnover of the various pairs of substrates and end products, the PP_i–ATP exchange occurs significantly slower than when promoted either by $tRNA^{Glu}$ or by Glu-$tRNA^{Glu}$ plus AMP, although under these conditions both pathways can occur. The decrease of the isotope-exchange rate under these conditions can be related to the formation of dead-end complexes resulting from the competition between $tRNA^{Glu}$ and Glu-$tRNA^{Glu}$ and between ATP and AMP for various enzyme forms:

$$E + Glu\text{-}tRNA^{Glu} + tRNA^{Glu} + ATP\cdot MgCl_2 + AMP + PP_i\cdot MgCl_2 + Glu \rightleftharpoons E\cdot Glu\text{-}tRNA^{Glu}\cdot ATP\cdot MgCl_2 + E\cdot tRNA^{Glu}\cdot AMP\cdot Glu \text{ (dead-end complexes)} + E\cdot Glu\text{-}tRNA^{Glu}\cdot AMP\cdot PP_i\cdot MgCl_2 + E\cdot tRNA^{Glu}\cdot ATP\cdot MgCl_2\cdot Glu \text{ (active complexes)}$$

The isotope exchange promoted by the active complexes is

$$E\cdot Glu\text{-}tRNA^{Glu}\cdot AMP\cdot PP_i\cdot MgCl_2 \rightleftharpoons E\cdot tRNA^{Glu}\cdot AMP\sim Glu\cdot PP_i\cdot MgCl_2 \rightleftharpoons E\cdot tRNA^{Glu}\cdot ATP\cdot MgCl_2\cdot Glu$$

Involvement of $tRNA^{Glu}$ in the Activation of Glutamate. Our results indicate an absolute requirement of uncharged $tRNA^{Glu}$ for the activation of glutamate (first step in eq 1). This is a general behavior for arginyl-, glutaminyl-, and glutamyl-tRNA synthetases of various organisms (Mehler & Mitra, 1967; Parfait & Grosjean, 1972; Nazario & Evans, 1974; Gangloff et al., 1976; Folk, 1971; Lapointe & Söll, 1972). Although kinetic studies performed on some of these systems indicated that during catalysis the substrates interact either in an ordered fashion, tRNA being first (Nazario & Evans, 1974), or in a random one (Papas & Peterkofsky, 1972; Charlier & Gerlo, 1979), binding studies always showed an absolute requirement of the cognate accepting tRNA for the binding of either one or two small substrates to the synthetase (Mitra et al., 1969; Parfait & Grosjean, 1972; Fersht et al., 1978; Kern & Lapointe, 1979). Thus, contrary to the other 17 tRNA charging systems, tRNA, besides its role of substrate, also acts as an effector in these systems. It can be that the correct positioning of the adenosine residue of the CCA end of tRNA promotes a conformational change of the synthetase, allowing the interaction of the small ligands; however, more complex mechanisms cannot be excluded.

Glutamyl-, glutaminyl-, and arginyl-tRNA synthetases are the smallest monomeric synthetases [in *E. coli*, respectively 56 000 (Kern et al., 1979), 69 000 (Folk, 1971; Kern et al., 1980), and 64 000 daltons (Craine & Peterkofsky, 1975)] which contain either no or much less sequence duplication compared to the monomeric synthetases of molecular weight ~100 000 [Kern et al. (1979, 1980) and general reviews by Söll & Schimmel (1974), Kisselev & Favorova (1974), and Schimmel & Söll (1979)]. It is interesting to correlate the fundamental monomerism of these three synthetases with their requirement of the cognate tRNA in the catalysis of the amino acid activation. Both properties are probably indicative of primitive structures.

Conclusion

As pointed out before, the results reported in this work do not allow us to exclude definitively the existence of a concerted mechanism for glutamyl-tRNA synthetase. Indeed the absolute $tRNA^{Glu}$ requirement for the [^{32}P]PP_i–ATP exchange and the much faster rate of this reaction compared to the rates of $tRNA^{Glu}$ charging and its reversal are also compatible with a one-step pathway if one postulates either the existence of an allosteric effect induced by the free $tRNA^{Glu}$ on the synthetase or the involvement of conformational changes occurring differently in the forward and in the reverse ways of the overall $tRNA^{Glu}$ charging process. Although tRNA effects on the isotope exchange have been reported for several tRNA charging systems [e.g., Von der Haar & Gaertner (1975), Von der Haar & Cramer (1978), and Kern & Giegé (1979)], there is no proven precedent for allosteric effects induced by substrates on these enzymes. Similarly, the second alternative seems also unlikely since such a kinetic situation was never reported for this class of enzymes. On the other hand, these kinetic properties can be better interpreted in light of a stepwise mechanism in which free $tRNA^{Glu}$ acts as an effector of the amino acid activation step. In this context the fast rate of isotope exchange as compared to the much slower ones of $tRNA^{Glu}$ charging and its reversal simply indicates that the first step occurs faster than the second one.

There is, in addition, further evidence for a stepwise mechanism for glutamyl-tRNA synthetase: (1) it catalyzes an equilibrated AMP-dependent and PP_i-independent deacylation of Glu-$tRNA^{Glu}$, (2) the initial rate of this reaction is similar to that of the overall reversal of $tRNA^{Glu}$ charging (the deacylation being AMP and PP_i dependent), and (3) the reversal of $tRNA^{Glu}$ charging generates an enzyme intermediate exhibiting properties similar to those of the enzyme·adenylates isolated for other synthetases. These results show that the reversal of $tRNA^{Glu}$ charging takes place in two chemical steps. The nature of the intermediate which is generated and its involvement in the overall $tRNA^{Glu}$ charging and the reversal are based on the argument of Jencks (1969) termed *generalization*: this intermediate has been characterized for other synthetases and its involvement in the tRNA charging established other tRNA charging systems, and all evidence is consistent with this for glutamyl-tRNA synthetase. We therefore believe that the mechanism of action of all synthetases is similar, and we strongly favor a two-step aminoacylation process for $tRNA^{Glu}$ charging.

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