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Glutamyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*. Study of the Interactions with Its Substrates†

Daniel Kern* and Jacques Lapointe

ABSTRACT: The binding of the various substrates to *Escherichia coli* glutamyl-tRNA synthetase has been investigated by using as experimental approaches the binding study under equilibrium conditions and the substrate-induced protection of the enzyme against its thermal inactivation. The results show that ATP and $tRNA^{Glu}$ bind to the free enzyme, whereas glutamate binds only to an enzyme form to which glutamate-accepting $tRNA^{Glu}$ is associated. By use of modified *E. coli* $tRNAs^{Glu}$ and heterologous $tRNAs^{Glu}$, a correlation could be established between the ability of $tRNA^{Glu}$ to be aminoacylated by glutamyl-tRNA synthetase and its abilities to promote the $[^{32}P]PP_i$–ATP isotope exchange and the binding of glutamate to the synthetase. These results give a possible explanation for the inability of glutamyl-tRNA synthetase to catalyze the isotope exchange in the absence of amino acid accepting $tRNA^{Glu}$ and for the failure to detect an enzyme·adenylate complex for this synthetase by using the usual approaches. One binding site was detected for each substrate. The specificity of the interaction of the various substrates has been further investigated. Concerning ATP, inhibition studies of the aminoacylation reaction by various analogues showed the existence of a synergistic effect between the adenine and the ribose residues for the interaction of adenosine. The primary recognition of ATP involves the N-1 and the 6-amino group of adenine as well as the 2′-OH group of ribose. This first interaction is then strengthened by the phosphate groups. Inhibition studies by various analogues of glutamate showed a strong decrease in the affinity of this substrate for the synthetase after substitution of the α- or γ-carboxyl groups. The enzyme exhibits a marked tendency to complex tRNAs of other specificities even in the presence of $tRNA^{Glu}$. $MgCl_2$ and spermidine favor the specific interactions. The influence of monovalent ions and of pH on the interaction between glutamyl-tRNA synthetase and $tRNA^{Glu}$ is similar to those reported for other synthetases not requiring their cognate tRNA to bind the amino acid. Finally, contrary to that reported for other monomeric synthetases, no dimerization of glutamyl-tRNA synthetase occurs during the catalytic process.

The aminoacyl-tRNA synthetases have very diverse structural and catalytic properties [for general reviews see Loftfield (1972), Kisselev & Favorova (1974), Söll & Schimmel (1974), Kalousek & Konigsberg (1975), Goddard (1977), and Ofengand (1977)]. No general properties of this class of enzymes could be defined except their ability to catalyze the aminoacylation of tRNAs.

The glutamyl-tRNA synthetase of *Escherichia coli* MRE-600 has been studied in several laboratories; two catalytically active forms have been described. This enzyme was first purified by Lapointe & Söll (1972a) and described as an $\alpha\beta$ dimer (M_r of α = 56000; M_r of β = 46000), where the α chain alone exhibited the synthetase activity, whereas the β chain was found able in vitro to modulate the tRNA charging activity. Modifying the purification procedure, we isolated a homogeneous monomeric enzyme of molecular weight 56000 (Kern et al., 1979), whose kinetic parameters were found the same as those of the previously described α chain. A similar form of glutamyl-tRNA synthetase has been described by Willick & Kay (1976) and by Powers & Ginsburg (1978).

The monomeric form of this synthetase is the smallest, among the native fully active aminoacyl-tRNA synthetases described so far, and, unlike the monomeric synthetases of molecular weight about 100000, does not contain significant sequence duplication (Kern et al., 1979).

This enzyme constitutes, with arginyl- and glutaminyl-tRNA synthetases, a particular family of synthetases characterized not only by their monomeric structure and their molecular weight significantly lower than 100000 but also by an absolute tRNA requirement for the catalysis of the $[^{32}P]PP_i$–ATP exchange and the inability to form an enzyme·adenylate complex in the presence of ATP and amino acid (Mehler & Mitra, 1967; Mitra & Smith, 1969; Folk, 1971; Parfait & Grosjean, 1972; Lapointe & Söll, 1972a; Nazario & Evans, 1974; Craine & Peterkofsky, 1975; Gangloff et al., 1976; Kern & Giegé, 1979). These particular kinetic behaviors have been used as major arguments in favor of a one-step aminoacylation mechanism for these synthetases (Loftfield, 1972). However, these properties could also reflect the inability of the three synthetases to bind one or both small substrates in the absence of tRNA and, therefore, cannot truly distinguish between a one-step and a two-step aminoacylation pathway.

In order to show if the particular kinetic properties of glutamyl-tRNA synthetase are related to an ordered association of the substrates, we here use two different approaches to investigate the binding of the various substrates to the enzyme either in the absence or in the presence of the other substrates. The results show that glutamate is unable to bind to the free enzyme, whereas ATP and $tRNA^{Glu}$ can bind. The binding of glutamate obligatorily requires the presence of accepting $tRNA^{Glu}$. The interactions of the substrates with the enzyme were then further investigated. The results are discussed in the context of the topology of the active center and the aminoacylation mechanism of this enzyme.

Experimental Procedures

General. $[^{14}C]$- and $[^{3}H]$-L-glutamate (specific activities, respectively, of 250 mCi/mmol and 25 Ci/mmol), $[^{14}C]$ATP (400 mCi/mmol), $Na[^{32}P]PP_i$, and omnifluor were purchased from New England Nuclear; unfractionated *E. coli* B tRNA and yeast tRNA (both containing 4% $tRNA^{Glu}$) were from Schwarz/Mann; 90% purified *E. coli* B $tRNA_2^{Glu}$ was from

† From the Département de Biochimie, Faculté des Sciences et de Génie, Université Laval, Québec, G1K7P4 Canada. *Received November 21, 1978; revised manuscript received September 18, 1979.* This work was supported by a grant (A-9597) to J.L. from the National Research Council of Canada.

* Permanent address and to which correspondence should be addressed: Institut de Biologie Moléculaire et Cellulaire du C.N.R.S., Laboratoire de Biochemie, 15, rue René Descartes, 67084 Strasbourg Cédex, France.

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Boehringer; 75% purified *Bacillus subtilis* $tRNA^{Glu}$ was prepared according to the procedure described by Vold (1971) and then further purified by reversed-phase chromatography (RPC5). Hepes,[1] L-glutamate, L-glutamine, γ-methyl-L-glutamate, γ-monohydroxamate L-glutamate, γ-ethyl-L-glutamate, α-methyl-L-glutamate, ATP (crystalline disodium salt), AMPcPP, AMP, ADP, adenosine, ribose, adenine, 2′-deoxy-ATP, 1-methyladenosine, inosine, GTP, CTP, UTP, and TTP were from Sigma Chemical Co. Poly(ethylene glycol) (molecular weight 6000) was from Fluka. Sephadex G-75 (fine) and dextran 500 (molecular weight 500 000) were from Pharmacia, microcapillaries were from Drummond Scientific Co., and cyanogen bromide was from Matheson Coleman and Bell. Nitrocellulose membranes (membrane filter 11 406, pore size 0.45 μm) were from Sartorius Gmbh. All other chemicals were of the highest purity commercially available.

The purity of the glutamate analogues was checked either by analysis with a Technicon amino acid analyzer or by chromatography on Whatman paper 3MM in the presence of phenol saturated with water as the solvent.

Purification of Glutamyl-tRNA Synthetase. The enzyme was purified as described previously (Kern et al., 1979) and had a turnover number of about 1.8 s^{-1} at pH 7.2 and 37 °C as tested by tRNA aminoacylation. Enzyme concentrations were determined from the absorbance (extinction coefficient $E_{mg/(mL\cdot cm)}^{280nm} = 0.87$) and by using a molecular weight of 56 000 (Kern et al., 1979).

Aminoacylation Reactions. The standard aminoacylation mixture contained 100 mM Na Hepes, pH 7.2, 2 mM ATP, 16 mM $MgCl_2$, 0.5 mM [^{14}C]-L-glutamate (30 000 cpm/nmol), 10 mg of unfractionated *E. coli* B tRNA per mL (15 μM $tRNA^{Glu}$), and an adequate concentration of enzyme (about 20 nM) if necessary before dilution in 10 mM Na Hepes, pH 7.2, 5 mM 2-mercaptoethanol, and 1 mg of bovine serum albumin per mL. After various incubation times at 37 °C the Glu-$tRNA^{Glu}$ synthesized was determined in samples as described previously (Kern et al., 1979). The kinetic constants K_m and K_i of the enzyme for ATP, glutamate, and their analogues were determined under similar conditions except that the free $MgCl_2$ concentration was held constant at 10 mM. The K_m for $tRNA^{Glu}$ was measured in the presence of 100 mM Na Hepes at the indicated pH, 2 mM ATP, 16 mM $MgCl_2$, 0.5 mM [^{3}H]-L-glutamate (650 cpm/pmol), and 0.37–1.3 μM $tRNA^{Glu}$. Special care was taken to select an enzyme concentration (0.48 nM) and incubation times (15 s–5 min) to obtain linear incorporations of glutamate into $tRNA^{Glu}$ at all pH values tested.

Glu-$tRNA^{Glu}$ was prepared on a large scale under standard aminoacylation conditions but increasing the volume of the incubation mixture and the enzyme concentration. The reaction was stopped after 30 min of incubation at 37 °C by addition of 100 mM sodium acetate, pH 4.5, and the [^{14}C]-Glu-$tRNA^{Glu}$ was isolated according to the methodology described by Yang & Novelli (1968).

[^{32}P]PP_i–ATP Isotope Exchange. The standard incubation mixture contained 100 mM Na Hepes, pH 7.2, 2 mM ATP, 16 mM $MgCl_2$, 2 mM Na[^{32}P]PP_i (1900 cpm/nmol), 1 mM L-glutamate, ATP or glutamate analogues when present at the concentrations indicated, 3 mM AMP (to reverse the aminoacylation of $tRNA^{Glu}$), 10 mg of unfractionated tRNA per mL and 15 nM enzyme. After various incubation times at 37 °C the [^{32}P]ATP synthesized was determined in samples as described previously (Lapointe & Söll, 1972a).

The K_m of $tRNA^{Glu}$ for glutamyl-tRNA synthetase was determined in the presence of either 100 mM Na Hepes, Na Mes, or glycine–NaOH buffer at the indicated pH, and 6 nM to 11 μM $tRNA^{Glu}$, the other reactants being present at the concentrations indicated for the standard medium. Special care was taken to select enzyme concentrations (1.8–7.1 nM) and incubation times (1–15 min) to obtain linear incorporations of [^{32}P]PP_i into ATP at all pH values tested.

Substrate-Induced Protection of Glutamyl-tRNA Synthetase against Heat Inactivation. Enzyme (10 nM–1.0 μM) was incubated at 50 °C in the presence of 100 mM Na Hepes, pH 7.2, 5 mM 2-mercaptoethanol, and 15 mM $MgCl_2$ either in the absence or in the presence of substrates ATP, glutamate, and $tRNA^{Glu}$ at the indicated concentrations. The inactivation process was stopped after 3, 5, and 10 min of incubation either by transferring the assay in an ethanol bath at −30 °C (when 10 nM enzyme was present) or by dilution of a sample of the mixture in cold buffer containing no substrate (when 1 μM enzyme was present). The remaining enzyme activity was then determined at 37 °C in the presence of the standard aminoacylation mixture.

Sucrose Gradient Centrifugations. The methodology was that described by Martin & Ames (1961). The 5–20% sucrose gradient contained 50 mM Na Hepes, pH 7.4, or Na Mes, pH 5.8, 15 mM $MgCl_2$ or spermidine trihydrochloride (as indicated), 5 mM 2-mercaptoethanol, ATP, glutamate, $tRNA^{Glu}$, and KCl as indicated in each case. The 100-μL samples of the buffer above described containing glutamyl-tRNA synthetase and the various substrates at the indicated concentrations were loaded on the top of the gradients. After centrifugation at 38 000 rpm at 4 °C for 16 h in a SW 50.1 rotor, 80-μL fractions were collected from the bottom of the gradients and assayed for the enzyme and/or the $tRNA^{Glu}$-accepting activities. Catalase was used to standardize the gradients and detected according to the procedure described by Chance & Maehly (1955).

Protein–Substrate Binding Measurements in a Two-Phase Aqueous Polymer System. We used the principle of the methodology described by Winlund-Gray & Chamberlain (1971). The 100-μL standard assay contained 7.5% poly(ethylene glycol) (molecular weight 6000), 7% dextran (molecular weight 500 000), 50 mM Na Hepes, pH 7.2, 0.1 mM dithioerythritol, 10 mM $MgCl_2$, glutamyl-tRNA synthetase, $tRNA^{Glu}$, ATP, and glutamate as indicated in each case. The incubation was carried out for 5 min at room temperature under vortex agitation. The mixtures were then transferred in 100-μL capillaries and centrifuged in a Hemato-kit Centrifuge Model HNS for 5 min at 1000 rpm until complete separation of the two phases. [^{14}C]ATP or [^{14}C]glutamate was determined in each phase by counting 10-μL samples in 10 mL of Bray's scintillator. When present, [^{14}C]Glu-$tRNA^{Glu}$ was measured as described above. The enzyme activity in each phase was measured in the presence of the standard aminoacylation mixture. No loss of activity was detected after 30 min of incubation of the enzyme in both phases at room temperature.

The partition coefficient K_p of glutamyl-tRNA synthetase (enzyme concentration in the poly(ethylene glycol) phase/enzyme concentration in the dextran phase) was 0.05. Those of ATP, glutamate, and $tRNA^{Glu}$ (or Glu-$tRNA^{Glu}$) determined in the absence of enzyme were, respectively, 0.706, 0.82, and 0.01 and were found unchanged in the presence of the other ligands.

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; BrCN, cyanogen bromide; EDTA, (ethylenedinitrilo)tetraacetate; AMPcPP, adenosine α,β-methylene-5′-triphosphate.

Treatment of the Data. As the enzyme accumulates mainly in the dextran-rich phase, only free ligand is present in the poly(ethylene glycol)-rich phase. The K_p value of the ligand and its concentration in the poly(ethylene glycol)-rich phase (C_{pg}) allows one to determine the free ligand concentration in the dextran-rich phase (C_d corresponding to $[ATP]_{free}$ or $[glutamate]_{free}$), according to $C_d = C_{pg}/K_p$. The concentrations of the enzyme-bound ligand ($[ATP]_{bound}$ or $[glutamate]_{bound}$) were obtained by the difference between the total ligand concentration (bound plus free) in the dextran-rich phase (determined by counting of a sample of this phase) and the free ligand concentration in this phase.

Binding of Glu-tRNA^{Glu} to Glutamyl-tRNA Synthetase Measured by Filtration through Nitrocellulose Disks. The incubation mixtures contained 100 mM Na Hepes, pH 7.2, 10 mM $MgCl_2$, 0.1 mg of bovine serum albumin per mL, 0.1 mM dithioerythritol, 0.035–1.37 μM [^{14}C]Glu-tRNA^{Glu} (142 cpm/pmol), and 0.12 μM glutamyl-tRNA synthetase. After 1 min of incubation in ice, 1-mL samples were filtered through nitrocellulose disks, washed 3 times with 2 mL of a buffer containing 100 mM Na Hepes, pH 7.2, 10 mM $MgCl_2$, 0.1 mg of bovine serum albumin per mL, and 0.1 mM dithioerythritol, dried, and counted. The yield of retention of the enzyme·Glu-tRNA^{Glu} complex determined in the presence of 4 μM enzyme and 0.5–2 μM [^{14}C]Glu-tRNA^{Glu} was 85%.

Modifications of tRNA^{Glu}. They were carried out either by periodate oxidation as described by Giegé et al. (1974) (except that only 1 mM sodium metaperiodate was present), which yields tRNA^{Glu} modified in the 3′-terminal ribose, or by cyanogen bromide treatment as described by Saneyoshi & Nishimura (1971), which specifically modifies more than 95% the 2-thiouridine derivative in the anticodon of tRNA^{Glu}.

Results

Attempts to Isolate a Glutamyl-tRNA Synthetase·Glutamyladenylate Complex. Glutamyl-tRNA synthetase catalyzes the [^{32}P]PP$_i$–ATP isotope exchange and the cleavage of [γ-^{32}P]ATP only in the presence of accepting tRNA^{Glu}; neither periodate-oxidized tRNA^{Glu} from *E. coli* nor tRNA^{Glu} from yeast or *B. subtilis* is able to promote these reactions (results not shown). We attempted the isolation of the enzyme·adenylate complex using the gel filtration methodology applied successfully for various other synthetases by Norris & Berg (1964) and by Allende et al. (1964). For glutamyl-tRNA synthetase, no enzyme·substrate complex could be isolated unless all the substrates were present in the gel-filtered incubation mixture. In the presence of all the substrates ([^{14}C]-glutamate and [γ-^{32}P]- or [α-^{32}P]ATP), only [^{14}C]Glu-tRNA^{Glu} was eluted with the enzyme. Neither free [^{14}C]-glutamate nor [^{32}P]ATP either α-^{32}P- or γ-^{32}P-labeled was detected in the macromolecular fractions eluted. In addition, the amounts of [γ-^{32}P]ATP cleaved and [^{32}P]PP$_i$ released both eluted in the micromolecular fractions were found equimolar to the Glu-tRNA^{Glu} synthesized (results not shown).

Interactions of Glutamyl-tRNA Synthetase with ATP and Glutamate. The interactions of ATP and glutamate with the enzyme were further investigated under equilibrium conditions by using the partition equilibrium method in a poly(ethylene glycol)–dextran two-phase system.

Binding of ATP. Figure 1A shows the saturation curves of glutamyl-tRNA synthetase by ATP in the absence and in the presence of either glutamate or tRNA^{Glu} or Glu-tRNA^{Glu} plus glutamate. The curves of enzyme·ATP complex concentration as a function of free ATP concentration are the same in the absence and in the presence of the other substrates. Thus, the dissociation constant of this system remains unchanged whether the other substrates are present or not. Figure 1B shows the Scatchard analysis of the saturation curve of the enzyme by ATP in the absence of the other ligands; the replot of the enzyme-bound ATP concentration over the free ATP concentration as a function of the enzyme-bound ATP concentration over the total enzyme concentration is linear and extrapolates to a point which indicates the existence of one binding site for ATP; from the slope a K_D value of 90 μM is determined (for further details see the legend to Figure 1).

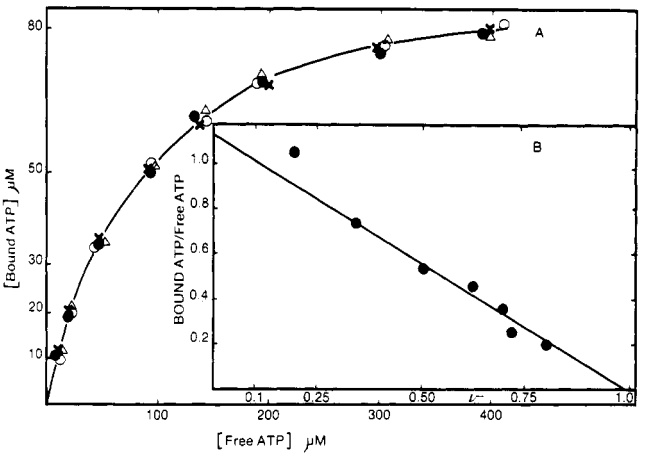

FIGURE 1: Binding of ATP to glutamyl-tRNA synthetase. (A) Saturation curves of the binding, studied in a poly(ethylene glycol)–dextran two-phase system. Enzyme (28.6 μM) (99 μM in the dextran phase and 5 μM in the poly(ethylene glycol) phase) was incubated in the absence of the other substrates (●) or in the presence of 1 mM L-glutamate (○) or 30 μM *E. coli* tRNA^{Glu} (×) or 30 μM unlabeled Glu-tRNA^{Glu} (△) and various concentrations of [^{14}C]ATP (40 cpm/pmol). The other conditions are described under Experimental Procedures. (B) Scatchard analysis of the saturation curve obtained in the absence of the other substrates and reported in Figure 1A: $[ATP]_{bound}/[ATP]_{free} = f(\nu)$, where ν = enzyme bound ATP/total enzyme in the dextran phase. Further details concerning the treatment of the data are given under Experimental Procedures.

Binding of Glutamate. Unlike ATP, glutamate binds to glutamyl-tRNA synthetase only in the presence of *E. coli* tRNA^{Glu} (Figure 2A). Periodate-oxidized *E. coli* tRNA^{Glu} or tRNA^{Glu} from yeast or *B. subtilis* was unable to promote this binding. However, modification of the 2-thiouridine group in the anticodon by cyanogen bromide did not affect the ability of tRNA^{Glu} to promote this binding. Finally, no binding was shown in the presence of ATP or in the presence of both ATP and tRNA^{Glu} (Figure 2A). Figure 2B shows the Scatchard analysis of the saturation curve of the enzyme by glutamate in the presence of tRNA^{Glu}. The replot of the enzyme-bound glutamate concentration vs. the free glutamate concentration as a function of the enzyme-bound glutamate concentration vs. the total enzyme concentration is linear and extrapolates to a point which indicates the existence of one binding site for glutamate. A K_D value of 180 μM for this system can be determined.

Binding of tRNA^{Glu} to Glutamyl-tRNA Synthetase. [^{14}C]Glu-tRNA^{Glu} was used for this study, and the enzyme·Glu-tRNA^{Glu} complex was separated from free Glu-tRNA^{Glu} by filtration through nitrocellulose disks. Figure 3 shows the Scatchard analysis of this binding experiment. At pH 7.2 one binding site and a K_D value of 0.09 μM were determined. The presence of the small substrates did not modify the dissociation constant of this complex.

Substrate-Induced Protection of Glutamyl-tRNA Synthetase against Heat Inactivation. The effects of tRNA^{Glu}, ATP, and glutamate upon enzyme inactivation at 50 °C have been studied and are described in another paper (Kern & Lapointe,

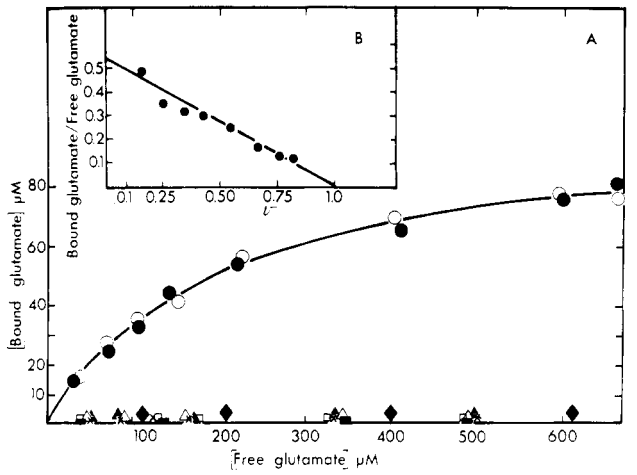

FIGURE 2: Binding of glutamate to glutamyl-tRNA synthetase. (A) Saturation curves of the binding, studied in a poly(ethylene glycol)–dextran two-phase system. Either 59.4 μM enzyme [198 μM in the dextran phase and 10 μM in the poly(ethylene glycol) phase] was incubated in the absence (×) or in the presence of 2 mM ATP (■) or 2 mM ATP and 30 μM *E. coli* $tRNA^{Glu}$ (◆) or 28.6 μM enzyme [99 μM in the dextran phase and 5 μM in the poly(ethylene glycol) phase] was incubated in the presence of 30 μM *E. coli* $tRNA^{Glu}$ (●) or yeast (▲) or *B. subtilis* (□) $tRNA^{Glu}$ or *E. coli* periodate-oxidized (△) or cyanogen bromide treated (○) $tRNA^{Glu}$ and various concentrations of [^{14}C]glutamate (34 cpm/pmol). The other conditions are described under Experimental Procedures. When $tRNA^{Glu}$ and ATP were present together, the [^{14}C]Glu-$tRNA^{Glu}$ synthesized and which accumulates in the dextran phase was determined by acido-precipitation of samples of the dextran phase. The [^{14}C]glutamate bound to the enzyme in the dextran phase was determined, taking into account the [^{14}C]Glu-$tRNA^{Glu}$ present. (B) Scatchard analysis of the saturation curve obtained in the presence of intact *E. coli* $tRNA^{Glu}$ reported in Figure 2A: $[glutamate]_{bound}/[glutamate]_{free} = f(\nu)$ where ν = enzyme-bound glutamate/total enzyme in the dextran phase. Further details concerning the treatment of the data are given under Experimental Procedures.

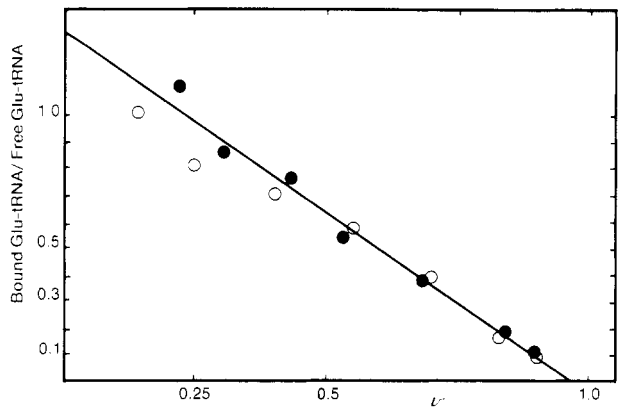

FIGURE 3: Binding of Glu-$tRNA^{Glu}$ to glutamyl-tRNA synthetase in the absence of the other substrates (○) or in the presence of 1 mM ATP and 1 mM L-glutamate (●). Scatchard analysis of the saturation curves (not reported): $[Glu\text{-}tRNA^{Glu}]_{bound}/[Glu\text{-}tRNA^{Glu}]_{free} = f(\nu)$. The conditions are described under Experimental Procedures, and ν is defined as enzyme-bound Glu-$tRNA^{Glu}$/total enzyme. The values of $[Glu\text{-}tRNA^{Glu}]_{bound}$ were corrected, taking into account the yield of retention of the enzyme·Glu-$tRNA^{Glu}$ complex on the nitrocellulose disks.

1979a). The most striking facts were the existence of two significant synergistic effects against enzyme inactivation: one occurring in the presence of $tRNA^{Glu}$ and glutamate and a second one occurring in the presence of the three substrates together. Table I shows that *E. coli* $tRNA^{Glu}$ and ATP were both efficient protectors of the enzyme: they induced, respectively, a protection of 39 and 11%, whereas glutamate did not. In the presence of $tRNA^{Glu}$, glutamate induced an additional protection of 46%. A weak synergistic effect occurred between ATP and $tRNA^{Glu}$: together they induced a protection of 51% which is slightly greater than the 44% expected from their separate effects. When the three substrates were present together, the observed protection (59%) was slightly

Table I: Effects of $tRNA^{Glu}$, ATP, and Glutamate on the Protection of Glutamyl-tRNA Synthetase against Heat Inactivation at 50 °C[a]

substrates			exptl values of protection (%)		
$tRNA^{Glu}$	ATP	Glu	protection (%)[d] (A)	expected[e] (B)	synergistic effect[g]
	–	–	0	–	
		+	0	–	
	+	–	11	–	
		+	11	11	
12 μM *E. coli* $tRNA^{Glu}$ [b]	–	–	39	–	
		+	67	39	+
	+	–	51	46	+–
		+	59	46	+
50 μM *E. coli* $tRNA^{Glu}$ [c]	–	–	39	39	
		+	67	39	+
	+	–	51	46	+–
		+	93	46	++
12 μM *E. coli* periodate-oxidized $tRNA^{Glu}$ [b]	–	–	39	39	
		+	39	39	–
	+	–	46	46	–
		+	46	46	–
50 μM *E. coli* BrCN-treated $tRNA^{Glu}$ [b]	–	–	39	39	
		+	67	39	+
	+	–	50	46	+–
		+	91[f]	46	++
12 μM yeast $tRNA^{Glu}$ [b]	–	–	39	39	
		+	39	39	–
	+	–	46	46	–
		+	47	46	–
12 μM *B. subtilis* $tRNA^{Glu}$ [b]	–	–	39	39	
		+	39	39	–
	+	–	46	46	–
		+	46	46	–

[a] Either 1 μM or 0.01 μM glutamyl-tRNA synthetase was incubated at 50 °C in the absence or in the presence of intact or modified *E. coli* $tRNA^{Glu}$ or yeast or *B. subtilis* $tRNA^{Glu}$ at the indicated concentrations, 4 mM ATP, and/or 1 mM L-glutamate for 3, 5, and 10 min, and the remaining enzyme activities were determined as indicated under Experimental Procedures. In the absence of substrates the remaining enzyme activity after 10 min of incubation was 15%. It was shown that in all cases the enzyme inactivations were first order. [b] 1 μM glutamyl-tRNA synthetase. [c] 0.01 μM glutamyl-tRNA synthetase. [d] The experimental values of protection (P_S) induced by one or several substrates together were determined from the percent of remaining enzyme activities in the absence (A_E) and in the presence ($A_{E,S}$) of this (these) substrate(s) at a given time according to $(A_{E,S}) = A_E + P_S - A_E P_S$. [e] The percents of protections expected were determined assuming that no synergistic effect occurred, i.e., that the resulting protection effect P_{S_1,S_2} induced by two substrates S1 and S2 present together was related to the protections P_{S_1} and P_{S_2} induced by each substrate separately according to $P_{S_1,S_2} = P_{S_1} + P_{S_2} - P_{S_1}P_{S_2}$. In addition, when a third substrate S3 inducing a protection P_{S_3} was present, the resulting protection P_{S_1,S_2,S_3} was given by $P_{S_1,S_2,S_3} = P_{S_1,S_2} + P_{S_3} - P_{S_1,S_2}P_{S_3}$, P_{S_1,S_2} being the protection induced by S1 and S2 when present together. [f] Under these conditions no significant accumulation of Glu-$tRNA^{Glu}$ occurred during the incubation time, since cyanogen bromide treated $tRNA^{Glu}$ is aminoacylated about 20 times slower than intact $tRNA^{Glu}$. [g] A synergistic protecting effect occurred in the presence of two or three substrates if the experimental value of protection shown in the presence of these substrates (A) was significantly higher than that expected (B). The amplitude of the synergistic effect is indicated by – (no synergistic effect) and +–, +, and ++ (increasing synergistic effects).

Table II: Relationship between the Glutamate-Accepting and the Isotope-Exchange Stimulation Capacities of Various $tRNAs^{Glu}$ in the Presence of *E. coli* Glutamyl-tRNA Synthetase and Their Abilities to Promote the Glutamate-Induced Protection of the Enzyme against the Heat Inactivation and the Binding of Glutamate to the Enzyme

$tRNA^{Glu}$	stimulation of the		stimulation of the interaction of the glutamate with the enzyme tested by	
	aminoacylation reaction	$[^{32}P]PP_i$–ATP exchange reaction	protection against heat inactivation	binding studies
E. coli intact $tRNA^{Glu}$	+	+	+	+
E. coli Glu-$tRNA^{Glu}$	–	–	–	–
E. coli periodate-oxidized $tRNA^{Glu}$	–	–	–	–
E. coli BrCN-treated $tRNA^{Glu}$	+	+	+	+
yeast $tRNA^{Glu}$	–	–	–	–
B. subtilis $tRNA^{Glu}$	–	–	–	–

Table III: Effects of Various Analogues of ATP on the Aminoacylation Reaction Catalyzed by Glutamyl-tRNA Synthetase[a]

ATP or analogue	rel rate of aminoacylation (%) 0.1 mM ATP	0.2 mM ATP	K_m[b] (mM)	K_i[b] (mM)	inhibition pattern
ATP	100	100	0.18		
2′-deoxy-ATP	–	–	6.0		
ribose (30 mM)	100	100			
adenine (30 mM)	100	100			
1-methyladenosine (10 mM)	96	100			
inosine (10 mM)	91	100			
GTP (10 mM)	77	84			
adenosine (10 mM)	57	65		8.9	competitive
ADP (10 mM)	35	42		3.6	competitive
AMP (10 mM)	23	24		3.0	noncompetitive
AMPcPP (10 mM)	6	8		0.45	competitive

[a] The experimental conditions are described under Experimental Procedures. [b] The concentrations of ATP varied from 50 to 400 μM, and those of [^{14}C]-L-glutamate and $tRNA^{Glu}$ were of 0.5 mM and 10 μM, respectively. The initial velocities were analyzed according to Lineweaver & Burk (1934).

greater than that expected taking into account the protection induced by each substrate separately (46%) but was significantly lower assuming that all synergistic effects occurred (71%). However, under these catalytic conditions, the $tRNA^{Glu}$ was aminoacylated in less than 1 min, so that the enzyme was mainly saturated by Glu-$tRNA^{Glu}$ instead of $tRNA^{Glu}$ during the incubation: indeed, the turnover number of the synthetase for tRNA charging is 1.55 s^{-1} and the half-life of Glu-$tRNA^{Glu}$ is 15 min in these experimental conditions. When the enzyme was incubated at 50 °C under catalytic conditions not allowing a significant accumulation of Glu-$tRNA^{Glu}$ during the incubation, a higher protection was observed (93%) than expected taking into account the synergistic effects induced by the various combinations of ligands (71%).

The synergistic effect between $tRNA^{Glu}$ and the small ligands for the protection of glutamyl-tRNA synthetase against heat inactivation has been investigated further. The influence of various modifications of *E. coli* $tRNA^{Glu}$ as well as the replacement of this tRNA by tRNAs of the same specificity but from other organisms has been studied. Table I shows that neither periodate oxidation nor cyanogen bromide treatment affected the ability of *E. coli* $tRNA^{Glu}$ to protect glutamyl-tRNA synthetase against heat inactivation. The protection also remained unaffected by replacement of *E. coli* $tRNA^{Glu}$ by yeast or *B. subtilis* $tRNA^{Glu}$. The slight synergistic effect observed between $tRNA^{Glu}$ and ATP remained after substitution of intact *E. coli* $tRNA^{Glu}$ by cyanogen bromide treated $tRNA^{Glu}$ but was less in the presence of periodate-oxidized or heterologous $tRNAs^{Glu}$. Among the various $tRNAs^{Glu}$ tested, only unmodified or cyanogen bromide treated *E. coli* $tRNA^{Glu}$ was able to promote the glutamate-induced protection of glutamyl-tRNA synthetase against heat inactivation. Similarly, the synergistic effect occurring under catalytic conditions (when all substrates were present and no significant accumulation of end products occurred) also remained unchanged after substitution of *E. coli* $tRNA^{Glu}$ by cyanogen bromide treated $tRNA^{Glu}$. Periodate-oxidized *E. coli* $tRNA^{Glu}$, as well as yeast and *B. subtilis* $tRNA^{Glu}$, was unable to promote these effects. Table II summarizes the various properties of the $tRNAs^{Glu}$ studied. A correlation appears between the ability of $tRNA^{Glu}$ (1) to be aminoacylated by glutamyl-tRNA synthetase, (2) to promote the $[^{32}P]PP_i$–ATP exchange, and (3) to promote the binding of glutamate to glutamyl-tRNA synthetase and the glutamate-induced protection of the enzyme against thermal inactivation.

Interaction of Glutamyl-tRNA Synthetase with Analogues of ATP and of Glutamate. Analogues of ATP. The contribution of the various components of the ATP molecule (adenine, ribose, and phosphate moieties) to its interaction with glutamyl-tRNA synthetase was studied in two sets of experiments. First, we attempted to stimulate the glutamylation process by other nucleoside triphosphates such as GTP, UTP, CTP, TTP, 2′-deoxy-ATP, and AMPcPP; second, we tested the inhibition of this reaction by adenine, ribose, adenosine (and two of its substituted analogues: 1-methyladenosine and inosine), AMP, and ADP. The results are given in Table III. All nucleoside triphosphates tested except 2′-deoxy-ATP could not promote the aminoacylation of $tRNA^{Glu}$; GTP and AMPcPP inhibited the reaction. 2′-Deoxy-ATP partially replaced ATP. The K_m of glutamyl-tRNA synthetase for this ATP analogue was about 30 times higher than for ATP, whereas the maximal rate of the aminoacylation reaction was about 45 times less: the rate constants of the aminoacylation reaction were 1.77 and 0.04 s^{-1} in the presence of saturating concentrations of ATP and 2′-deoxy-ATP, respectively. Among the various components of the ATP molecule tested, adenine and ribose did not inhibit even at very high concentrations the aminoacylation reaction, whereas adenosine, 1-methyladenosine, inosine, AMP, and ADP did inhibit. Furthermore, adenosine inhibited the aminoacylation less than AMP and ADP but more than 1-methyladenosine and inosine. Finally, adenosine, AMPcPP, and ADP were competitive inhibitors, whereas AMP was a noncompetitive inhibitor with respect to ATP. The K_i values of the various ATP analogues are given for Table III.

Analogues of Glutamate. Five analogues varying in their substitution either on the α-carboxyl group (α-methylglutamate) or on the γ-carboxyl group (γ-methyl-, γ-monoethyl-, and γ-hydroxamate glutamate and glutamine) were inhibitors of the aminoacylation reaction (Table IV). In order to exclude artifacts due to isotope dilution of the [^{14}C]-glutamate by contaminating unlabeled glutamate in these

Table IV: Effects of Various Analogues of Glutamate on the [^{32}P]PP_i–ATP Exchange and on the Aminoacylation Reactions Catalyzed by Glutamyl-tRNA Synthetase

glutamate or analogue	rel rates (%) of amino-acylation[a]	isotope exchange[b]	K_i (mM)[c]
L-glutamate	100	100	
γ-methyl-L-glutamate	55	60	3.1
α-methyl-L-glutamate	82	85	12.0
L-glutamine	91	93	28.0
γ-monohydroxamate L-glutamate	94	95	37.8
γ-ethyl-L-glutamate	94	96	42.0

[a] The aminoacylation reactions were conducted in the presence of 0.1 mM L-glutamate and 5 mM analogue. [b] The isotope-exchange reactions were conducted in the presence of 0.4 mM L-glutamate and 5 mM analogue. The other conditions are described under Experimental Procedures. [c] The initial velocities of aminoacylation were analyzed and the K_i values were determined according to Lineweaver & Burk (1934); the concentrations of L-glutamate varied from 5 to 77 μM (K_m = 0.1 mM); a competitive inhibition pattern with respect to glutamate was observed for all analogues.

analogues, we tested their purity (1) by chromatography (cf. Experimental Procedures) and (2) by testing their effect on the [^{32}P]PP_i–ATP exchange. No glutamate contaminations were detected by chromatographic analysis. In addition, all these analogues were inhibitors of the isotope exchange (Table IV), indicating more certainly the absence of significant glutamate contaminations. The five analogues are competitive inhibitors with respect to glutamate in the aminoacylation reaction; their K_i values are given in Table IV. γ-Methylglutamate was the most efficient inhibitor.

Complexes between Glutamyl-tRNA Synthetase and tRNA^{Glu} in the Absence and in the Presence of the Small Substrates. Glutamyl-tRNA synthetase sedimented in a 5–20% sucrose gradient with an apparent molecular weight of 51 200 and a sedimentation coefficient of 4.0 S (by reference to the catalase: molecular weight 240 000, s = 11.3 S, Figure 4A). In the presence of a stoichiometric or even a much higher tRNA^{Glu} concentration, the synthetase sedimented with tRNA^{Glu} as a complex of apparent molecular weight 107 000 and a sedimentation coefficient of 11.3 S. These characteristics suggest the existence in this complex of a 1:1 stoichiometry between glutamyl-tRNA synthetase (apparent molecular weight 51 200, s = 4.0 S) and tRNA^{Glu} (apparent molecular weight 55 000, s = 4.24 S; Figure 4B,C). This stoichiometric ratio was further revealed by testing the enzyme and tRNA^{Glu} present in the pooled fractions of the peak of the complex (not shown). tRNA^{Glu} sedimented in the absence of $MgCl_2$ (EDTA present) with an apparent molecular weight of 55 000 (s = 4.24 S) and in the presence of $MgCl_2$ with an apparent molecular weight of 76 900 (s = 5.3 S) (Figure 4D). Using similar conditions, Yaniv & Gros (1969) determined an apparent molecular weight of *E. coli* tRNA^{Val} of 55 000. Furthermore, Eisinger & Grass (1975) reported the existence of dimers of tRNA^{Glu} in the presence of $MgCl_2$. Consequently, these different tRNA^{Glu} behaviors probably indicate the formation of dimers of tRNA^{Glu} in our experimental conditions. Finally, when glutamyl-tRNA synthetase was sedimented under equilibrium conditions (all substrates present along the gradient), the apparent molecular weight of the enzyme·substrate complex was very similar to that of the enzyme·tRNA^{Glu} complex (Figure 4E).

Specificity of the Interaction between Glutamyl-tRNA Synthetase and tRNA^{Glu}. When centrifuged in the presence of various concentrations of unfractionated *E. coli* tRNA,

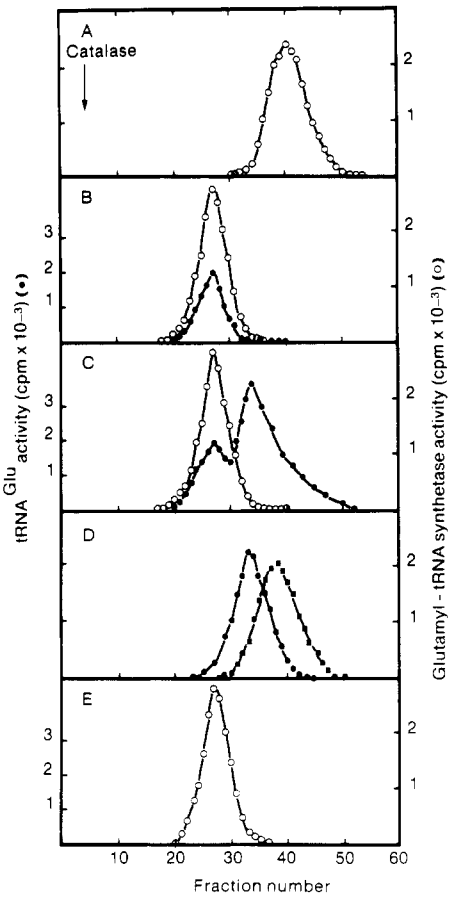

FIGURE 4: Centrifugation of glutamyl-tRNA synthetase on a sucrose gradient at pH 5.8 in the absence and in the presence of the various substrates. (A–C) Glutamyl-tRNA synthetase (250 pmol) was centrifuged under the conditions described under Experimental Procedures in the absence (A) or in the presence of 250 (B) or 1000 pmol (C) of purified tRNA^{Glu}; (D) 700 pmol of tRNA^{Glu} was centrifuged either in the presence of 15 mM $MgCl_2$ (●) or in the absence of $MgCl_2$ but in the presence of 10 mM EDTA (■); (E) 250 pmol of glutamyl-tRNA synthetase was centrifuged in a sucrose gradient equilibrated with 10 μM tRNA^{Glu}, 2 mM ATP, and 1 mM L-glutamate.

glutamyl-tRNA synthetase sedimented in a single peak with characteristics of the enzyme·tRNA^{Glu} complex (apparent molecular weight 107 300, s = 6.6 S), even when less tRNA^{Glu} than enzyme was present (Figure 5A). Increasing unfractionated tRNA concentrations increased the tRNA^{Glu}/glutamyl-tRNA synthetase ratio in this peak (Figure 5A–C). When tRNA^{Glu} was present in a fourfold excess with respect to glutamyl-tRNA synthetase, a 1:1 stoichiometric complex was obtained; the presence of higher tRNA concentrations did not modify this stoichiometry (results not shown). The free tRNA^{Glu} sedimented mainly as a dimer. These results suggest that in these experimental conditions (1) the monomeric and the dimeric forms of tRNA^{Glu} are present in unfractionated tRNA, only the monomeric form being able to bind to the enzyme, and (2) glutamyl-tRNA synthetase interacts with tRNAs of other specificities.

Effect of KCl on the Association between Glutamyl-tRNA Synthetase and tRNA^{Glu}. The presence of increasing KCl concentrations in the sucrose gradient lowered the sedimentation coefficient of the enzyme·tRNA^{Glu} complex, which was correlated with a progressive dissociation of the enzyme and tRNA^{Glu} activities (results not shown). When centrifuged in the presence of 0.8 M KCl at pH 7.4, glutamyl-tRNA synthetase and tRNA^{Glu} sedimented in two large peaks largely overlapping (Figure 5D). The major part of the enzyme and

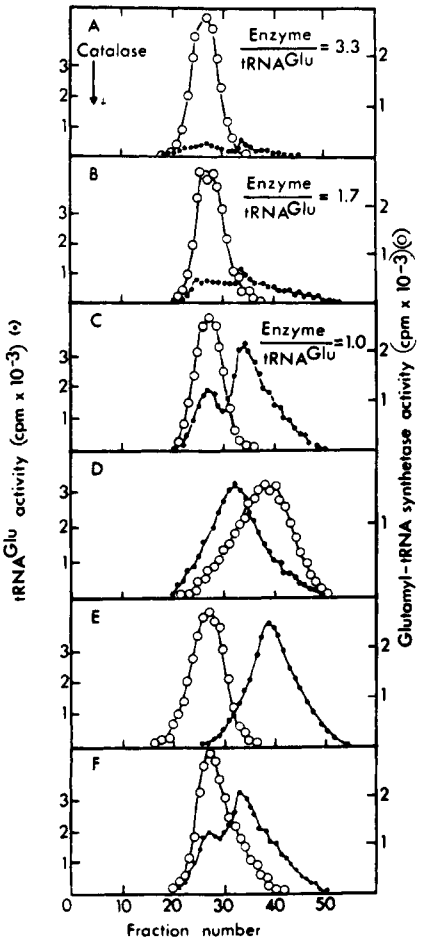

FIGURE 5: Centrifugation of glutamyl-tRNA synthetase in the presence of unfractionated *E. coli* tRNA under various ionic strength and pH conditions. (A–C) Glutamyl-tRNA synthetase (250 pmol) was centrifuged at pH 5.8 under the conditions described under Experimental Procedures in the presence of 130 (A), 350 (B), or 1000 pmol (C) of $tRNA^{Glu}$ in unfractionated *E. coli* tRNA. The enzyme/$tRNA^{Glu}$ ratios in the peaks of complex are indicated in each case. (D–F) Glutamyl-tRNA synthetase (250 pmol) and $tRNA^{Glu}$ (1000 pmol) in unfractionated *E. coli* tRNA were centrifuged either in the presence of Na Hepes buffer, pH 7.4, and 0.8 M KCl (D) or in the presence of Na Mes buffer, pH 5.8, in the absence of added $MgCl_2$ (E and F) or in the presence of 15 mM spermidine (F).

the $tRNA^{Glu}$ molecules sedimented under these conditions with an apparent molecular weight of, respectively, 55 000 (s = 4.23 S) and 84 800 (s = 5.65 S). However, both were still present in the region of the gradient where the complex was previously found.

Effects of Magnesium and Spermidine on the Association between Glutamyl-tRNA Synthetase and $tRNA^{Glu}$. When centrifuged in the presence of unfractionated tRNA and in the absence of added $MgCl_2$, glutamyl-tRNA synthetase sedimented in a peak with characteristics of the enzyme·$tRNA^{Glu}$ complex (apparent molecular weight 107 300, s = 6.6 S), but no $tRNA^{Glu}$ was present in this peak (Figure 5E). Under these conditions $tRNA^{Glu}$ sedimented in its monomeric form (apparent molecular weight 55 000, s = 4.24 S). In the presence of spermidine (instead of $MgCl_2$) $tRNA^{Glu}$ was again associated to glutamyl-tRNA synthetase, both sedimenting in a peak of apparent molecular weight 107 300 (s = 6.6 S), while the free $tRNA^{Glu}$ sedimented in its dimeric form (s = 5.3 S) (Figure 5F).

pH Dependence of the Affinity of Glutamyl-tRNA Synthetase for $tRNA^{Glu}$. This study was effected kinetically. We determined the K_m values of $tRNA^{Glu}$ in the aminoacylation and the $[^{32}P]PP_i$–ATP exchange reactions as a function of pH. The experimental conditions and the results are given in Table V. In both reactions the K_m of glutamyl-tRNA synthetase for $tRNA^{Glu}$ increased with increasing pH.

Table V: Effect of pH on the K_m of $tRNA^{Glu}$ for Glutamyl-tRNA Synthetase in the Aminoacylation and the $[^{32}P]PP_i$–ATP Isotope-Exchange Reactions[a]

pH	buffer	$K_m \times 10^{-8}$ (M) amino-acylation	$K_m \times 10^{-8}$ (M) $[^{32}P]PP_i$–ATP exchange
5.5	Mes		2.7
6.2	Mes	7.1	3.0
6.8	Hepes		6.6
7.2	Hepes	12.3	8.1
7.6	Hepes		10.1
8.0	Hepes	15.2	13.2
8.6	Hepes		31.0
9.1	Hepes		66.6
9.4	glycine-NaOH		92.4
9.8	glycine-NaOH		120.3

[a] The experimental conditions are described under Experimental Procedures. Particular cares were taken to obtain linear initial velocities at each pH (see Experimental Procedures) and to ensure saturation by the nonvaried substrate. [K_m for glutamate: 80 μM at pH 6.2; 110 μM at pH 7.4 and 8.6. K_m for ATP: 100 μM at pH 6.2; 150 μM at pH 7.4; 250 μM at pH 8.6. K_m for pyrophosphate: 50 μM at pH 6.2; 700 μM at pH 9.8 (as determined for the isotope-exchange reaction.)]

Discussion

Binding of Glutamate to Glutamyl-tRNA Synthetase Requires the Presence of Accepting $tRNA^{Glu}$. The experiments reported here show a good correlation between the results obtained by the two approaches used to study the association of the various substrates to glutamyl-tRNA synthetase. Indeed, $tRNA^{Glu}$ and ATP are both able to bind to the free enzyme, whereas glutamate does not; on the other hand, $tRNA^{Glu}$ and ATP both efficiently protect the enzyme against heat inactivation, whereas in the absence of the other substrates, glutamate does not protect it. The $tRNA^{Glu}$-promoted binding of glutamate makes the enzyme thermally more stable. Chuang et al. (1967) and Chuang & Bell (1972) demonstrated the possibility of using protection against thermal denaturation to measure binding of ligands to aminoacyl-tRNA synthetases. The protection constants of the various substrates we determined for glutamyl-tRNA synthetase are similar to the dissociation constant values reported here (Kern & Lapointe, 1979a). Thus, the same enzyme·substrate(s) complex(es) are monitored in each case.

Our results indicate that $tRNA^{Glu}$ induces a change of glutamyl-tRNA synthetase, rendering it able to bind glutamate. By use of various approaches, tRNA-induced changes affecting various parameters such as structural parameters, association constants of the small ligands, or even kinetic constants of the $[^{32}P]PP_i$–ATP isotope exchange have mainly been described for synthetases not requiring their tRNA to catalyze the isotope exchange (Goldstein & Holley, 1960; Loftfield & Eigner, 1965; Hele & Barth, 1966; Buonocore & Schlesinger, 1972; Ostrem & Berg, 1974; Di Natale et al., 1974; Fasiolo & Ebel, 1974; Krauss et al., 1975, 1977; Hustedt et al., 1977; Jacques & Blanquet, 1977; Zaccai et al., 1979; Kern & Giegé, 1979). Among the synthetases which require their cognate tRNA to catalyze the isotope exchange, arginyl-tRNA synthetase has been most studied, and tRNA effects on the binding of small ligands to this enzyme have been reported. Mitra et al. (1969) described a tRNA-dependent protection induced by arginine against the heat inactivation

of *E. coli* arginyl-tRNA synthetase. On the basis of these results and others (Mitra & Mehler, 1966), these authors suggested that $tRNA^{Arg}$ induces a catalytically active form of this enzyme. Parfait & Grosjean (1972) proposed on the basis of binding experiments that $tRNA^{Arg}$ in combination with ATP induces a conformational change on arginyl-tRNA synthetase from *Bacillus stearothermophilus*, rendering it able to bind arginine. Finally, Fersht et al. (1978) showed that yeast arginyl-tRNA synthetase binds arginine only in the presence of $tRNA^{Arg}$. Concerning the *E. coli* glutamyl-tRNA synthetase, we provide some information about the regions of $tRNA^{Glu}$ responsible for the structural change in the enzyme allowing the binding of glutamate. As periodate-oxidized $tRNA^{Glu}$, Glu-$tRNA^{Glu}$, and heterologous $tRNAs^{Glu}$ do not promote the binding of glutamate, it appears that the integrity of the 3′-OH end as well as an adequate positioning of this extremity of $tRNA^{Glu}$ on the enzyme is required to induce this change. The modification of the 5-(methylaminomethyl)-2-thiouridine residue in the anticodon of $tRNA^{Glu}$ by cyanogen bromide does not affect its ability to promote the binding of glutamate to the synthetase, indicating that the anticodon region of $tRNA^{Glu}$ is not required for the interaction of glutamate with the synthetase. These results establish a correlation between the amino acid acceptance capacity of $tRNA^{Glu}$ and its ability to promote the binding of glutamate to glutamyl-tRNA synthetase. As, however, the modification of $tRNA^{Glu}$ by cyanogen bromide results in a decrease in the rate of $tRNA^{Glu}$ charging (Kern & Lapointe, 1979b), without affecting its ability to promote the binding of the glutamate to the synthetase, this correlation is not direct.

In addition to the tRNA-induced change of the enzyme, there are other changes induced by the association of the small ligands and which result in an increase of the thermal stability of the enzyme: the first occurs after the association of $tRNA^{Glu}$ and ATP and another one occurs when the three substrates are present together on the enzyme. These changes lead progressively the free inactive enzyme form to the quaternary catalytically active enzyme·$tRNA^{Glu}$·ATP·glutamate complex. This complex, or another one involved in the catalytic process, is strongly protected against the thermal inactivation. A strong synergistic effect on the thermal stability of arginyl-tRNA synthetase from *B. stearothermophilus* induced by the three substrates together was also reported by Parfait (1973a).

Topology of the Binding Sites of ATP and Glutamate. Glutamyl-tRNA synthetase is highly specific for ATP. Of the various nucleoside triphosphates tested only 2′-deoxy-ATP stimulates the aminoacylation of $tRNA^{Glu}$. Similar results were reported for prolyl-, lysyl-, isoleucyl-, and tyrosyl-tRNA synthetases (Mitra & Mehler, 1969; Zamecnick et al., 1966; Santi & Pena, 1971), for arginyl-tRNA synthetases (Hirschfield & Bloemers, 1969), and for phenylalanyl-tRNA synthetases (Santi et al.,1971). The strong increase of the K_m (30-fold) and the decrease of the V_{max} (about 45-fold) when ATP is substituted by 2′-deoxy-ATP indicate that the 2′-OH group of the ribose residue contributes to the interaction of ATP with glutamyl-tRNA synthetase. Inhibition studies show the existence of a significant synergistic effect between adenine and ribose in the interaction of adenosine with the enzyme. Thus, the association of both components despite the reduction of their degree of liberty results in a molecule able to interact more strongly with the synthetase than the two components separately can. As 1-methyladenosine and inosine are weaker inhibitors than adenosine, either a substitution on N-1 or the exchange of the 6-amino residue by an OH residue significantly affects the interaction of adenosine with the enzyme. The introduction of a substitute on N-1 produces not only a steric modification in the adenosine molecule but also a major modification in the distribution of the charges in the adenine residue. So, the substitution on N-1 of hydrogen by a methyl group makes an imino tautomer predominate in position 6 (Simon et al., 1970), correlated with a decrease of the pK_a value to 8.2 (Macon & Wolfenden, 1968). Similar results were obtained for *E. coli* methionyl-tRNA synthetase (Lawrence et al., 1974) and *Salmonella typhimurium* histidyl-tRNA synthetase (Di Natale et al., 1976). AMP, ADP, and AMPcPP, compared with adenosine, are strong inhibitors of the aminoacylation reaction, indicating that the phosphate groups contribute largely to the interaction of ATP with the enzyme. As GTP inhibits the reaction less efficiently than do ADP, AMP, and AMPcPP, it appears that the primary interaction occurs with the nucleoside group, the phosphate groups strengthening then this interaction. The competitive inhibition by ADP and AMPcPP with respect to ATP and the noncompetitive one by AMP indicate that in addition to its effect as a dead-end inhibitor as are ADP and AMPcPP, AMP acts at another level, probably by reversing the reaction. Similar kinetic behaviors of ADP and AMP were reported for the *S. typhimurium* histidyl-tRNA synthetase (Di Natale et al., 1976).

The study of the inhibition of the aminoacylation reaction by several analogues of glutamate shows a high specificity of glutamyl-tRNA synthetase for this substrate. Various substitutions either on the α- or on the γ-carboxyl groups decrease the affinity of this ligand for the synthetase 44–650-fold, indicating that the two carboxyl groups of glutamate participate in its interaction with the enzyme. The strong decrease of the affinity after substitution of a hydrophilic group (carboxyl) by a hydrophobic one (methyl or ethyl) suggests that these interactions are ionic and/or hydrophilic. However, as glutamine and γ-hydroxamate glutamate (both resulting from a substitution of the α- or the γ-carboxyl group by another hydrophilic one) also react weakly with the enzyme, the ionic interactions probably prevail in this association. The poor inhibitory effect of α-methyl glutamate indicates that in addition to the direct involvement of this residue in the catalysis it also significantly contributes to the interaction of the amino acid with the enzyme.

Complex of Glutamyl-tRNA Synthetase with Its Substrates during the Catalytic Process. Sucrose gradient centrifugation studies as well as binding experiments show that glutamyl-tRNA synthetase and $tRNA^{Glu}$ combine in a 1:1 stoichiometric complex. These results agree with previous studies of this system (Lapointe & Söll, 1972b). The dissociation constant value of this system is in the range of those reported for other synthetase–tRNA systems (Loftfield, 1972; Kisselev & Favorova, 1974; Söll & Schimmel, 1974, Kalousek & Konigsberg, 1975; Goddard, 1977; Ofengand, 1977).

In the presence of ATP and glutamate, the sedimentation coefficient of the glutamyl-tRNA synthetase·$tRNA^{Glu}$ complex remains unchanged. Thus, (1) the binding of ATP and glutamate does not trigger a major conformational change of the glutamyl-tRNA synthetase·$tRNA^{Glu}$ complex and (2) the enzyme does not dimerize during the catalysis. This result differs from that reported by Parfait (1973b) for the arginyl-tRNA synthetase from *B. stearothermophilus*. Indeed, in the presence of similar conditions, this synthetase sediments as a dimer (i.e., a 2:1 stoichiometric enzyme·$tRNA^{Arg}$ complex). tRNA-induced dimerizations were also observed for the yeast lysyl- (Österberg et al., 1975) and valyl-tRNA

synthetases (Giegé et al., 1977) by using X-ray and neutron small-angle scattering approaches. However, no evidence for an obligatory dimerization of these synthetases during the catalytic process has been uncovered. Our results provide strong evidence for the existence of a monomeric catalytically active form of glutamyl-tRNA synthetase.

Specificity of the Interaction of Glutamyl-tRNA Synthetase with tRNA^{Glu}. In the presence of unfractionated *E. coli* tRNA, aspecific interactions occur between glutamyl-tRNA synthetase and tRNAs of other specificities. Indeed, the enzyme sediments quantitatively complexed to tRNA even when less tRNA^{Glu} (in unfractionated tRNA) than glutamyl-tRNA synthetase is present. However, as under these conditions free tRNA^{Glu} remains present, and as increasing unfractionated tRNA concentrations increase the amount of enzyme·tRNA^{Glu} complex in the total enzyme·tRNA complex, no simple competition occurs between tRNA^{Glu} and tRNAs of other specificities for glutamyl-tRNA synthetase. Indeed, such competition would result in a constant ratio between the specific enzyme·tRNA^{Glu} and the aspecific enzyme·tRNA complexes for increasing unfractionated tRNA concentrations. As the remaining free tRNA^{Glu} sediments as a dimer, these tRNA-binding properties of glutamyl-tRNA synthetase could be related to the formation of stable tRNA^{Glu} dimers unable to bind to the synthetase, making easier the association of noncognate tRNAs.

In the presence of EDTA, glutamyl-tRNA synthetase combines only with incorrect tRNAs. $MgCl_2$ and spermidine favor the specific interactions. These results agree with those obtained by kinetic studies showing that the aminoacylation and the [^{32}P]PP$_i$–ATP isotope exchange reactions catalyzed by this synthetase both require a significantly higher $MgCl_2$/ATP ratio (8 at the optimum) than other tRNA-acylating or amino acid activating systems (Loftfield, 1972; Kisselev & Favorova, 1974; Söll & Schimmel, 1974). Thus, the tRNA^{Glu} requires an exceptionally high $MgCl_2$ concentration (as compared to tRNAs of other specificities) to acquire the structure able to interact with the synthetase. Furthermore, both reactions are able to be stimulated by spermidine in the presence of limiting $MgCl_2$ concentrations (results not shown). As spermidine does not replace $MgCl_2$ in the formation of the reactive ATP·$MgCl_2$ complex (Holler, 1973), these stimulations result probably from the substitution of the $MgCl_2$ by spermidine in the productive association of tRNA^{Glu} with the synthetase. Similar results were obtained for *E. coli* valyl-tRNA synthetase (Chakraburtty et al., 1975).

Effects of Monovalent Ions and pH on the Stability of the Glutamyl-tRNA Synthetase·tRNA^{Glu} Complex. When centrifuged together in the presence of 0.8 M KCl at pH 7.4, glutamyl-tRNA synthetase and tRNA^{Glu} sediment heterogeneously in two peaks overlapping greatly. The major part of both components sediment with a higher apparent molecular weight than when sedimented individually. This results from a dynamic interaction between glutamyl-tRNA synthetase and tRNA^{Glu} in the experimental conditions used. Indeed, the salt-induced increase of the association and dissociation rate constants of the complex decreases its stability, resulting in a heterogeneous behavior of the various components during the centrifugation. The competition effect of monovalent ions has been studied for other tRNA–synthetase interacting systems, and strong salt-induced increases of the dissociation constants of these systems have been reported (Bonnet & Ebel, 1975).

Increasing pH values increase the K_m of glutamyl-tRNA synthetase for tRNA^{Glu} in the aminoacylation and the [^{32}P]PP$_i$–ATP exchange reactions. As the rate constant of tRNA^{Glu} charging increases with increasing pH (Kern and Lapointe, unpublished experiments), the pH-induced increase of the K_m of tRNA^{Glu} in this reaction could be related to the increase of the rate constant. However, in the [^{32}P]PP$_i$–ATP isotope exchange, tRNA^{Glu} acts as an activator (Kern & Lapointe, 1979b), indicating that in this reaction the K_m values can be assimilated to the dissociation constant values of the glutamyl-tRNA synthetase·tRNA^{Glu} complex. As a consequence, increasing pH values, although being favorable for the tRNA^{Glu} charging, decrease the affinity of tRNA^{Glu} for the synthetase. The decrease in the stability for increasing pH has also been reported for other synthetase–tRNA interacting systems (Loftfield, 1972; Bonnet & Ebel, 1975).

Conclusion

The investigation of the binding of the various substrates to glutamyl-tRNA synthetase presented here does not allow us to discriminate between a two-step or a one-step aminoacylation pathway. However, in the viewpoint of a two-step pathway, the results allow us to propose a possible explanation for the absolute tRNA requirement for the catalysis of the [^{32}P]PP$_i$–ATP exchange by this synthetase. Indeed, as glutamate cannot bind in the absence of tRNA^{Glu}, the amino acid cannot be activated unless all substrates are present. The three substrates were generally found able to bind individually to the free form of the aminoacyl-tRNA synthetases able to catalyze the amino acid activation in the absence of tRNA, although kinetic studies often showed ordered associations of the substrates in the catalytic processes. For these synthetases, ping-pong mechanisms where ATP and amino acid bind either randomly or in an ordered fashion (generally ATP first and amino acid second) before PP$_i$ dissociates and tRNA interacts have been described [for general reviews see Söll & Schimmel (1974) and Kisselev & Favorova (1974)]. Glutamyl-tRNA synthetase, as well as arginyl- and glutaminyl-tRNA synthetases, catalyzes the aminoacylation of its tRNA via a sequential mechanism: no end product is formed unless all substrates are bound to these synthetases. For *E. coli* arginyl-tRNA synthetase, a random association of the substrates during the catalytic process has been reported by Papas & Peterkofsky (1972) and by Charlier & Gerlo (1979), although direct interaction experiments suggested that arginine binds to the synthetase only in the presence of tRNA^{Arg} (Mitra et al., 1969). Ordered associations of substrates, tRNA always as first, were also proposed for the aminoacylation reactions catalyzed by arginyl-tRNA synthetase from *B. stearothermophilus* (Parfait & Grosjean, 1972; Parfait, 1973b) and from *Neurospora crassa* (Nazario & Evans, 1974). The experiments reported here indicate that under the experimental conditions used, glutamate does not bind to glutamyl-tRNA synthetase in the absence of accepting tRNA^{Glu}, whereas ATP does. However, these results give no accurate information about the order of association of the substrates during the glutamylation process. Kinetic studies are in progress to elucidate this mechanism.

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