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ARTICLE *in* BIOCHEMISTRY · JANUARY 1980

Impact Factor: 3.02 · DOI: 10.1021/bi00593a011 · Source: PubMed

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Glutamyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*. Effect of Alteration of the 5-(Methylaminomethyl)-2-thiouridine in the Anticodon of Glutamic Acid Transfer Ribonucleic Acid on the Catalytic Mechanism†

Daniel Kern* and Jacques Lapointe

ABSTRACT: The 5-(methylaminomethyl)-2-thiouridine group in the anticodon of *Escherichia coli* tRNA^{Glu} has been specifically modified by cyanogen bromide in conditions where all modified molecules remained homogeneous and did not lose their aminoacylation ability. Modification reduced the steady-state rate of tRNA^{Glu} charging about 20-fold, so that incomplete aminoacylation plateaus were obtained as a function of the enzyme concentration. These plateaus reflected an equilibrium between the aminoacylation of modified tRNA^{Glu} and the chemical deacylation of Glu-tRNA^{Glu}. The kinetics of aminoacylation of modified tRNA^{Glu} were biphasic: after completion of the first turnover of the enzyme, the rate of aminoacylation decreased about fourfold. No decrease in the rate of intact tRNA^{Glu} charging occurred after the first turnover of the enzyme. These results indicate that the modification of the 2-thiouridine derivative in the anticodon of tRNA^{Glu} displaces the rate-determining step of the overall glutamylation process and are discussed in the light of a transconformation step taking place between the catalysis of Glu-tRNA^{Glu} synthesis and the dissociation of the end products. Moreover, this modification resulted in a fivefold decrease of the maximal rate of Glu-tRNA^{Glu} synthesis (the rate in the first turnover of the enzyme), whereas the rate of the tRNA^{Glu}-promoted [^{32}P]PP$_i$–ATP isotope exchange remained unaffected. This indicates that neither tRNA^{Glu} nor Glu-tRNA^{Glu} is an obligatory substrate of the isotope exchange and favors a two-step aminoacylation pathway for the action of glutamyl-tRNA synthetase.

The thiolated uridine residues present in *Escherichia coli* tRNAs have been widely used as a probe for investigating the structural and functional properties of the tRNAs. It was first shown that the 4-thiouridine residue at position 8 in some *E. coli* tRNAs can be cross-linked to the cytidine-13 in the D arm, demonstrating the close proximity of these two residues in the conformation of these tRNAs (Favre et al., 1969). Furthermore, spin-labeling of the 5-(methylaminomethyl)-2-thiouridine at the 5′ end of the anticodon of *E. coli* tRNA$_2^{Glu}$ allowed Caron et al. (1976) to show a conformational change of the anticodon region of this tRNA induced by its aminoacylation. In addition, circular dichroism measurements showed a displacement to longer wavelengths of the absorbance band of this residue after the association of tRNA^{Glu} with glutamyl-tRNA synthetase (Willick & Kay, 1976), indicating that this residue plays a role in the interaction between the two macromolecules.

Since 5-(methylaminomethyl)-2-thiouridine is the only thiolated base in tRNA$_2^{Glu}$, chemical modifications by cyanogen bromide were undertaken to study its role in the interaction between tRNA^{Glu} and glutamyl-tRNA synthetase. Saneyoshi & Nishimura (1971) showed a significant decrease of the acceptor capacity of tRNA^{Glu} after treatment of unfractionated *E. coli* tRNA by this sulfhydryl reagent. Agris et al. (1973) and Seno et al. (1974), who used various approaches of modifying this 2-thiouridine derivative in tRNA$_2^{Glu}$, reported (1) that tRNA^{Glu} extracted from *E. coli* cells grown in a sulfur-deficient medium can be aminoacylated at the same rate and to the same extent as tRNA^{Glu} extracted from cells grown in a standard medium and (2) that modification of the 2-thiouridine derivative of tRNA^{Glu} by cyanogen bromide reduces the rate and the extent of aminoacylation of this tRNA and increases the K_m of the tRNA for the synthetase about 10-fold. These results were used as an evidence for the involvement of the 2-thiouridine derivative at the 5′ end of the anticodon in determining the kinetic parameters of the glutamylation system.

In the present work, we reinvestigate the effect of modification by cyanogen bromide of the thiolated uridine residue of *E. coli* tRNA$_2^{Glu}$ on the kinetic constants of the aminoacylation and the [^{32}P]PP$_i$–ATP isotope-exchange reactions. A relevant interpretation of such a study involving a modified tRNA implies that all modified tRNA molecules are homogeneous and that the kinetic constants are measured at the same stage of the overall catalytic process; in other words, if these constants are compared at the steady state, the rate-determining step should not be modified in the presence of the modified tRNA, a statement which cannot a priori be assumed. Thus, we have demonstrated the homogeneity of the modified tRNA molecules and investigated the nature of the rate-determining step and found it different in the native and the modified aminoacylation systems. Consequently, kinetic constants cannot be compared at the steady state, since they are a resultant of kinetic parameters which are affected differently in both systems. Finally, no effect of the ability of tRNA^{Glu} to promote the [^{32}P]PP$_i$–ATP isotope exchange was detected after its modification. These results show that tRNA modifications can be used to detect partial stages which would otherwise be unnoticed in the overall aminoacylation process; they are discussed in the context of the catalytic mechanism of glutamyl-tRNA synthetase.

Experimental Procedures

General. Glutamyl-tRNA synthetase was purified to homogeneity from *E. coli* MRE 600 as described previously

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0006-2960/79/0418-5819$01.00/0

(Kern et al., 1979). *E. coli* $tRNA_2^{Glu}$ (accepting 1350 pmol of glutamate per A_{260nm} unit) was a generous gift from Dr. A. D. Kelmers of the Oak Ridge National Laboratory. [^{14}C]- and [^{3}H]-L-glutamate (specific activities, respectively, 250 mCi/mmol and 25 Ci/mmol), Na[^{32}P]PP_i, and omnifluor were obtained from New England Nuclear; ATP (crystalline disodium salt), Mes, Hepes, and L-glutamate were purchased from Sigma Chemical Co. Cyanogen bromide was obtained from Matheson Coleman and Bell. Norit, from Fisher, was activated by a 48-h treatment with HCl, 1 N, and then washed with doubly distilled water until neutral. GF/c glass fiber disks were from Whatman.

Determination of the Enzyme Concentrations. Protein concentrations were determined from the absorbance at 280 nm with an extinction coefficient ($E_{mg/(mL\cdot cm)}^{280nm}$) of 0.87 and a molecular weight of 56 000 (Kern et al., 1979).

Aminoacylation of $tRNA^{Glu}$. The 300-μL reaction mixture contained 100 mM Na Hepes,[1] pH 7.2, or 100 mM Na Mes, pH 6.2, 2 mM ATP, 16 mM $MgCl_2$, 0.4 mM L-glutamate labeled either with ^{14}C (30–150 cpm/pmol) or ^{3}H (650 cpm/pmol), $tRNA_2^{Glu}$ or BrCN-treated $tRNA_2^{Glu}$, and glutamyl-tRNA synthetase in concentrations as indicated in each case. The reactions were effected either at 37 °C (at pH 7.2) or at 0 °C (at pH 6.2), and the [^{14}C]- or [^{3}H]Glu-$tRNA^{Glu}$ was synthesized after various incubation times determined in 20- or 50-μL samples as described previously (Kern et al., 1979).

The K_m and V_{max} values for intact and BrCN-treated $tRNA_2^{Glu}$ were determined at 0 °C and pH 6.2, the other conditions being the same as those described above. When intact $tRNA^{Glu}$ was tested, 0.041–0.55 μM $tRNA_2^{Glu}$ and 0.78 nM enzyme were present. When modified $tRNA^{Glu}$ was tested, 1–100 μM $tRNA_2^{Glu}$ and 0.22 μM enzyme were present.

[^{32}P]PP_i–ATP Isotope-Exchange Reaction. The 300-μL reaction mixture contained 100 mM Na Mes, pH 6.2, 2 mM Na[^{32}P]PP_i (52 400 cpm/nmol), 16 mM $MgCl_2$, and 1 mM L-glutamate, either 0.05–0.5 μM intact $tRNA^{Glu}$ or 2–14.3 μM BrCN-treated $tRNA^{Glu}$, and 0.18 μM enzyme (when intact $tRNA^{Glu}$ was tested) or 0.36 μM enzyme (when BrCN-treated $tRNA^{Glu}$ was tested). The reactions were conducted at 0 °C, and the amounts of [^{32}P]ATP synthesized after various incubation times were determined in 50-μL samples as described previously (Kern et al., 1979).

Modification of $tRNA^{Glu}$ by Cyanogen Bromide. The reaction was carried out according to the procedure described by Saneyoshi & Nishimura (1970, 1971). Approximately 1 A_{260nm} unit of $tRNA_2^{Glu}$ in 0.1 mL of 100 mM $NaHCO_3$ (pH 8.9) was shaken with 250 μg of BrCN for 10 min at room temperature. The solution was then adjusted to pH 6.0 and dialyzed against 0.5 mM Na_2EDTA at 4 °C for 14 h.

Results

Aminoacylation Kinetics of Intact and BrCN-Treated $tRNA^{Glu}$ in the Presence of Catalytic Concentrations of Glutamyl-tRNA Synthetase. Comparative kinetic experiments in the presence of the same concentration of intact and modified $tRNA^{Glu}$ (0.66 μM) and a catalytic enzyme concentration (0.36 nM) showed that the modified $tRNA^{Glu}$ was significantly more slowly aminoacylated than the intact one (the apparent rate constants k_a were, respectively, 0.032 and 1.86 s^{-1}). Furthermore, no biphasicity was found in the kinetics of aminoacylation of modified $tRNA^{Glu}$ which would indicate the presence of contaminating intact $tRNA^{Glu}$ (charged at a faster rate than modified $tRNA^{Glu}$) in the modified $tRNA^{Glu}$ preparation. This agrees with the chromatographic analysis effected by Agris et al. (1973) showing that, in the experimental conditions used, more than 95% of the $tRNA_2^{Glu}$ molecules were specifically modified in the 2-thiouridine derivative of the 5′ end of the anticodon. When the kinetics were conducted in the presence of about 20 times more $tRNA^{Glu}$ (12 μM), the rate of aminoacylation of BrCN-treated $tRNA^{Glu}$ became higher (k_a = 0.093 s^{-1}), whereas that of intact $tRNA^{Glu}$ remained unchanged (k_a = 1.90 s^{-1}). Increasing further the concentration of modified $tRNA^{Glu}$ did not modify the rate of aminoacylation. These results confirm the previous reports of Agris et al. (1973) and Seno et al. (1974) according to which the BrCN-induced modification of the 2-thiouridine derivative in the anticodon of $tRNA^{Glu}$ increases its K_m for glutamyl-tRNA synthetase.

Dependence of the Extent of Aminoacylation of BrCN-Treated $tRNA^{Glu}$ upon Glutamyl-tRNA Synthetase Concentration and Checking the Homogeneity of the Modified $tRNA^{Glu}$ Preparation. When the modified $tRNA^{Glu}$ was charged in the presence of increasing enzyme concentrations, increasing rates of aminoacylation were shown, and after about 60 min incomplete aminoacylation plateaus were obtained, corresponding to increasing aminoacylation extents for increasing enzyme concentrations (Figure 1A). In the presence of the same enzyme concentrations, intact $tRNA^{Glu}$ was 100% charged (Figure 1A). The incomplete aminoacylation extents of modified $tRNA^{Glu}$ did not result from an inactivation of the enzyme during the incubation, since the specific activity of the enzyme remained unchanged during the incubation in the aminoacylation mixture containing modified $tRNA^{Glu}$ (Figure 1B).

The replot of the aminoacylation data of Figure 1A in the form $1/[E]$ as a function of $[tRNA^{Glu}]_0/[Glu\text{-}tRNA^{Glu}]$, where [E] and $[tRNA^{Glu}]_0$ are, respectively, the concentrations of the enzyme and of $tRNA^{Glu}$ present at the beginning of the reaction (at $t = 0$) and $[Glu\text{-}tRNA^{Glu}]$ is the concentration of Glu-$tRNA^{Glu}$ present at the plateau, shows a linear relationship between $[E]^{-1}$ and $[tRNA^{Glu}]_0/[Glu\text{-}tRNA^{Glu}]$ (Figure 1C). This linear relationship is like those studied in the yeast correct and incorrect valylation systems (Bonnet & Ebel, 1972; Dietrich et al., 1976; Giegé et al., 1978) and in the *E. coli* isoleucylation system (Yarus, 1972a–c; Rich & Schimmel, 1977). Thus, the incomplete aminoacylation plateaus obtained in the presence of BrCN-treated $tRNA^{Glu}$ reflect the existence of an equilibrium between the aminoacylation reaction and various deacylation reactions; the chemical deacylation and the enzymatic deacylations are, respectively, AMP and PP_i dependent and independent. The kinetic behavior of this system can be used to test the homogeneity of the modified $tRNA^{Glu}$ preparation. Indeed, if all $tRNA^{Glu}$ molecules are chargeable at the same rate, the $[tRNA^{Glu}]_0/[Glu\text{-}tRNA^{Glu}]$ ratio will be defined (Bonnet & Ebel, 1972; Dietrich et al., 1976) by

$$\frac{[tRNA^{Glu}]_0}{[Glu\text{-}tRNA^{Glu}]} = \frac{k_b(K_m + [tRNA^{Glu}]_0}{k_a}\frac{1}{[E]} + \frac{k_c + k_d}{k_a} + 1 \quad (1)$$

where k_a, k_b, k_c, and k_d are, respectively, the rate constants of aminoacylation, chemical deacylation, and enzymatic deacylation, respectively AMP and PP_i independent and dependent, and K_m is the Michaelis constant of modified

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

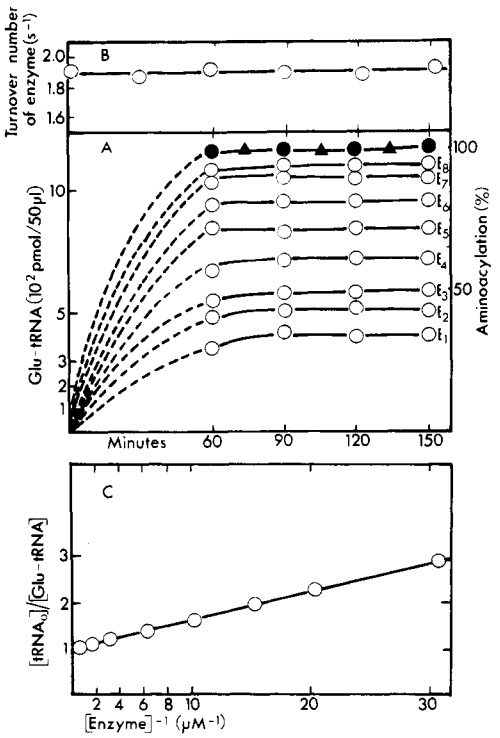

FIGURE 1: Kinetics of aminoacylation of BrCN-treated tRNA^{Glu} in the presence of various concentrations of glutamyl-tRNA synthetase. (A) The experiments were conducted at pH 7.2 and 37 °C in the presence of 23 µM BrCN-treated tRNA^{Glu} (○) or tRNA^{Glu} (●, ▲). The enzyme concentrations were as follows: E_1, 0.0325 µM; E_2, 0.0490 µM; E_3, 0.0653 µM; E_4, 0.0980 µM; E_5, 0.160 µM; E_6, 0.326 µM; E_7, 0.653 µM; E_8, 1.63 µM. In the presence of intact tRNA^{Glu}, 0.0325 (●) or 1.63 µM (▲) enzyme was present. The other conditions are described under Experimental Procedures. (B) Determination of the turnover of glutamyl-tRNA synthetase after various incubation times in the aminoacylation medium containing modified tRNA^{Glu}. A complete aminoacylation medium containing 1.63 µM enzyme, modified tRNA^{Glu}, and the other substrates as described in (A) was incubated at 37 °C. At various times, samples were withdrawn and diluted in a buffer containing 100 mM Na Hepes, pH 7.2, 0.1 mg of bovine serum albumin per mL, and 0.2 mM dithioerythritol at 0 °C. The turnovers of the enzyme were then estimated by initial rate measurements in the standard aminoacylation medium at 37 °C. (C) Replot of $1/[E] = f([tRNA]_0/[Glu\text{-}tRNA])$ of the data of the kinetics shown in (A): [E] = concentration of glutamyl-tRNA synthetase; $[tRNA]_0$ = concentration of BrCN-treated tRNA^{Glu} at $t = 0$; [Glu-tRNA] = concentration of modified Glu-tRNA^{Glu} at the plateau.

tRNA^{Glu} for the synthetase. So, if the modified tRNA^{Glu} molecules are homogeneous, the slope of the line of Figure 1A should be calculable from eq 1. The use of eq 1 presumes the identity between the K_m for tRNA^{Glu} and K_i for Glu-tRNA^{Glu} in the aminoacylation system, which was actually verified in our system. Using the experimental values of $[tRNA]_0 = 23$ µM, $k_a = 0.093\ s^{-1}$, $K_m = 1.2$ µM, and $k_b = 2.42 \times 10^{-4}\ s^{-1}$ at 37 °C and pH 7.2, we calculated a value for this slope of 0.0629 µM, which is very similar to the experimental value of 0.0625 µM determined in Figure 1A. This identity demonstrates the homogeneity of the modified tRNA^{Glu} population and justifies the further experiments undertaken with this tRNA. Both enzymatic Glu-tRNA^{Glu} deacylation reactions, AMP and PP_i dependent and independent, play a negligible role in the establishment of the aminoacylation plateaus since the straight line of Figure 1A extrapolates to a $[tRNA^{Glu}]_0/[Glu\text{-}tRNA^{Glu}]$ ratio of 1.0. This extrapolation also means that the BrCN-treated tRNA^{Glu} is able to be 100% charged, provided that a sufficiently high enzyme concentration is present. We emphasize that the incomplete aminoacylation extents of BrCN-treated tRNA^{Glu} are not due to the

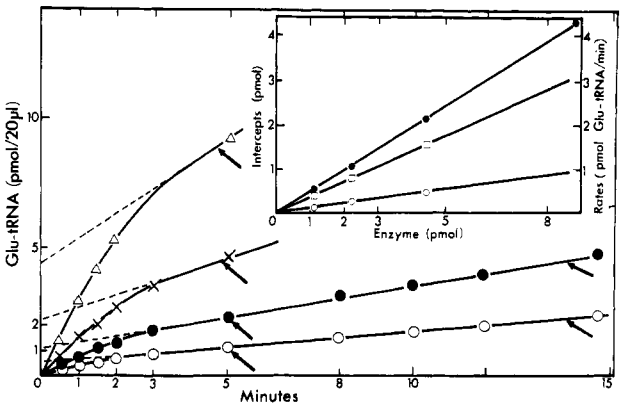

FIGURE 2: Kinetics of aminoacylation of BrCN-treated tRNA^{Glu} in the presence of various high concentrations of glutamyl-tRNA synthetase and a fixed concentration of modified tRNA^{Glu}. (A) The experiments were conducted at pH 6.2 (Mes buffer) and 0 °C in the presence of 50 µL of BrCN-treated tRNA^{Glu} and the following glutamyl-tRNA synthetase concentrations: 0.055 (○); 0.11 (●); 0.22 (×); 0.44 µM (△); the other conditions are described under Experimental Procedures. The arrows indicate the successive catalytic cycles of the enzyme. The insert figure shows the replots of the experimental data of these kinetics. (1) Intercept = f([enzyme]) (●); the intercepts correspond to the amounts of glutamyl-tRNA synthesized in a 20-µL sample during the fast phase and obtained by extrapolation of the slow phase to $t = 0$; the [enzyme] corresponds to the amount present in a 20-µL sample. (2) Rate(fast phase) = f([enzyme]) (□). (3) Rate(slow phase) = f([enzyme]) (○).

presence of inactive molecules unable to be charged but reflect the contribution of the hydrolysis of Glu-tRNA^{Glu} during the incubation.

Aminoacylation Kinetics in the Presence of High Glutamyl-tRNA Synthetase Concentrations. At pH 6.2 and 0 °C the steady-state rate constant of aminoacylation was sufficiently reduced (about 55 times compared with that at pH 7.2 and 37 °C) so that the first catalytic cycles of tRNA charging can be followed. At saturating substrate concentrations [2 mM ATP ($K_m = 10^{-4}$ M), 0.4 mM L-glutamate ($K_m = 0.08$ mM), and 50 µM modified tRNA^{Glu} ($K_m = 3$ µM as shown below)], the aminoacylation kinetics were biphasic, the rate decreasing continuously from the beginning of the reaction until the end, where there was a constant value (Figure 2). In the presence of increasing enzyme concentrations (0.055–0.44 µM), the rates of aminoacylation of both phases increased, and both were linear functions upon the enzyme concentration (Figure 2, insert). The amount of Glu-tRNA^{Glu} synthesized during the fast phase (and obtained by extrapolation of the linear slow phase to $t = 0$) was also a linear function of the amount of enzyme present (Figure 2, insert), the first being in a constant stoichiometric ratio of 0.62 with the second. Thus, the decrease of the rate of Glu-tRNA^{Glu} synthesis occurred from the beginning of the reaction until the end of the first catalytic cycle when the steady state of the overall reaction was established. Theoretical considerations about the meaning of the stoichiometry of 0.62 mol of Glu-tRNA^{Glu} synthesized during the first cycle of catalysis per mol of glutamyl-tRNA synthetase present are given in the Appendix. The rate constants of aminoacylation in the fast phase and the slow phase (the steady state) were, respectively, 0.6×10^{-2} and $0.17 \times 10^{-2}\ s^{-1}$.

Aminoacylation Kinetics in the Presence of Limiting BrCN-Treated tRNA^{Glu} Concentrations and a High Glutamyl-tRNA Synthetase Concentration. When the concentration of the modified tRNA^{Glu} was varied from 1 to 100 µM, the other ligands being present at saturating concentrations and the enzyme being present at a constant concentration, biphasic

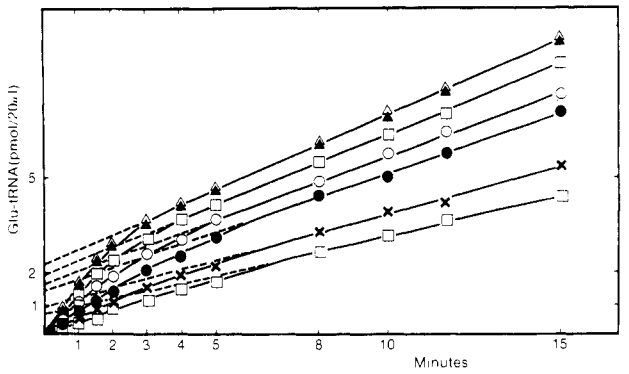

FIGURE 3: Kinetics of aminoacylation of BrCN-treated $tRNA^{Glu}$ in the presence of various concentrations of modified $tRNA^{Glu}$ and a fixed high concentration of glutamyl-tRNA synthetase. The experiments were conducted at pH 6.2 (Mes buffer) and at 0 °C in the presence of 0.22 µM enzyme and the following concentrations of BrCN-treated $tRNA^{Glu}$: 1 (□); 1.5 (×); 3 (●); 5 (○); 10 (□); 50 (▲); 100 µM (△). The other conditions are described under Experimental Procedures.

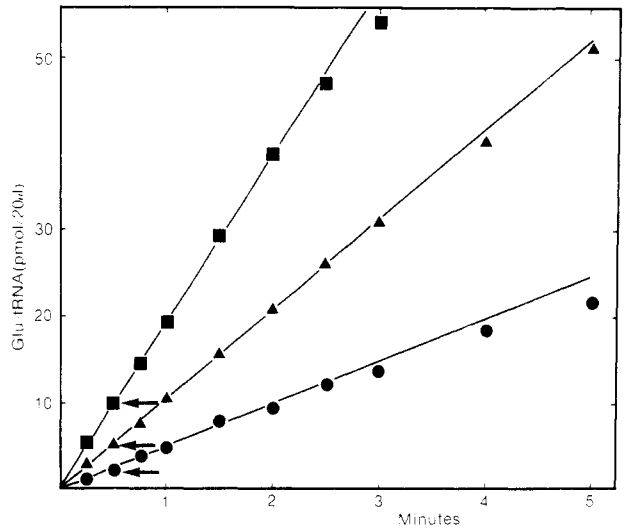

FIGURE 4: Kinetics of aminoacylation of intact $tRNA^{Glu}$ in the presence of high concentrations of glutamyl-tRNA synthetase. The experiments were conducted at pH 6.2 (Mes buffer), at 0 °C, in the presence of 14.4 µM $tRNA^{Glu}$ and the following concentrations of glutamyl-tRNA synthetase: 0.125 (●); 0.250 (▲); 0.500 µM (■). The arrows indicate the end of the first catalytic cycle.

kinetics were also obtained: the amounts of Glu-$tRNA^{Glu}$ synthesized during the fast phase increased with increasing concentrations of modified $tRNA^{Glu}$ (Figure 3). However, the aminoacylation rate in the first phase increased until an optimal value was reached when about 50 µM modified $tRNA^{Glu}$ was present, and neither this rate nor the amount of Glu-$tRNA^{Glu}$ synthesized during this phase was proportional to the initial concentration of modified $tRNA^{Glu}$. Thus, these bursts could not be attributed to the presence of intact $tRNA^{Glu}$ contaminating the modified $tRNA^{Glu}$ preparation.

Aminoacylation Kinetics of Intact $tRNA^{Glu}$ in the Presence of High Glutamyl-tRNA Synthetase Concentrations. In the presence of aminoacylation conditions that allow one to follow the first catalytic cycles of aminoacylation (see conditions above), no decrease in the rate was observed after the first turnover of the enzyme (Figure 4).

Kinetic Parameters of Glutamyl-tRNA Synthetase for Intact and BrCN-Treated $tRNA^{Glu}$ in the Aminoacylation and in the [^{32}P]PP_i–ATP Exchange Reactions. The kinetic parameters of aminoacylation of modified $tRNA^{Glu}$ have been determined from the fast phase and the slow phase (steady state) of the kinetics and compared to those of aminoacylation of intact $tRNA^{Glu}$ (Table I). The maximal rate of charging of modified $tRNA^{Glu}$ is about fourfold slower at the steady state than during the first cycle of catalysis. In addition, the rate of aminoacylation of intact $tRNA^{Glu}$ is about 5-fold faster than that of modified $tRNA^{Glu}$ during the first catalytic cycle, whereas, at the steady state, intact $tRNA^{Glu}$ is aminoacylated about 20-fold faster than is modified $tRNA^{Glu}$. The maximal rate of the $tRNA^{Glu}$-promoted [^{32}P]PP_i–ATP isotope exchange remains unchanged either in the presence of intact or modified $tRNA^{Glu}$.

Table I: Kinetic Constants of the Aminoacylation and of the [^{32}P]PP_i–ATP Isotope-Exchange Reactions Catalyzed by Glutamyl-tRNA Synthetase in the Presence of Intact and BrCN-Treated $tRNA^{Glu}$

		reaction tested		
substrate	kinetic parameter	aminoacylation		[^{32}P]PP_i–ATP exchange
$tRNA^{Glu}$	K_m	0.073 µM[a]		0.033 µM[a]
	V_{max}	0.033 s^{-1} [a] 0.033 s^{-1} [b]		0.97 s^{-1} [a]
		fast phase	slow phase	
BrCN-treated $tRNA^{Glu}$	K_m	3.07 µM[a]	0.8 µM[a]	1.82 µM[a]
	V_{max}	0.0063 s^{-1} [a] 0.0061 s^{-1} [b]	0.0017 s^{-1} [a] 0.0018 s^{-1} [b]	0.96 s^{-1} [a]

[a] Values were determined according to Lineweaver & Burk (1934). The conditions are described under Experimental Procedures. [b] Values were determined from the kinetics shown in Figure 2 (in the presence of modified $tRNA^{Glu}$) and in Figure 4 in the presence of intact $tRNA^{Glu}$.

The K_m of modified $tRNA^{Glu}$ for the enzyme is about fourfold higher in the fast phase of aminoacylation than at the steady state. These K_m values are higher than for intact $tRNA^{Glu}$: indeed, the K_m values of intact and modified $tRNA^{Glu}$ for the synthetase are in a ratio of about 8 at the steady state, whereas during the first cycle of catalysis this ratio is 42. The K_m of $tRNA^{Glu}$ in the isotope exchange increases by a factor of about 50 after modification by BrCN. This increase is similar to that shown in the first catalytic cycle of aminoacylation. It appears also that the K_m values of intact and modified $tRNA^{Glu}$ in the first catalytic cycle of aminoacylation and in the isotope exchange are both in a ratio of about 2.

Discussion

The modification of the 2-thiouridine derivative in the anticodon of $tRNA_2^{Glu}$ by cyanogen bromide results in large modifications in the aminoacylation kinetics due to differential changes in the rate constants of the various steps involved in the overall aminoacylation process.

Existence of Incomplete Aminoacylation Plateaus. The strong decrease (about 20-fold) of the rate of aminoacylation after modification of $tRNA^{Glu}$ gives rise to incomplete aminoacylation plateaus as a consequence of the establishment of an equilibrium between the aminoacylation and the chemical deacylation reactions. The glutamyl-tRNA synthetase catalyzed deacylations do not participate significantly in this equilibrium. It is easy to explain why the reverse of tRNA charging does not contribute, assuming formation of stoichiometric concentrations of end products AMP, PP_i, and Glu-$tRNA^{Glu}$ (21 µM at the maximum in our experimental conditions) in the light of the respective affinities of AMP and PP_i for the enzyme (K_m for PP_i in the isotope exchange, 0.2 mM; K_i for AMP in the aminoacylation reaction, 3 mM) and

the relatively slow rate constant of this reaction (0.012 s^{-1} at pH 7.2 and 37 °C) compared to that of tRNA charging (0.093 s^{-1}). Bonnet & Ebel (1972) and Dietrich et al. (1976) reported also that this reaction did not contribute for other nonmodified incomplete aminoacylation systems.

However, varying behaviors were reported for the AMP- and PP_i-independent enzymatic deacylation according to the system studied. Glutamyl-tRNA synthetase catalyzes this reaction at a considerably slower rate than other systems such as yeast phenylalanyl- and valyl-tRNA synthetases (Dietrich et al., 1976). As a consequence, with modified tRNA^{Glu} only the chemical deacylation contributes to the incomplete extents of aminoacylation, whereas, in correct and incorrect yeast tRNA valylation and phenylalanylation systems, both the chemical and the enzymatic deacylations largely contribute to the incomplete aminoacylation plateaus (Bonnet & Ebel, 1972; Dietrich et al., 1976). However, this enzymatic deacylation plays a minor role in the extents of aminoacylation of turnip yellow mosaic virus RNA charging, where only the chemical deacylation contributes to the plateaus (Giegé et al., 1978).

The slow rate constants of the AMP- and PP_i-independent Glu-tRNA^{Glu} deacylation reaction reported in this study do not result from the modification of tRNA^{Glu}, since deacylation kinetics of intact Glu-tRNA^{Glu} effected under similar experimental conditions revealed also the lack of any appreciable enzymatic activity of this type (Kern and Lapointe, unpublished experiments). The obtaining of complete charging of intact tRNA^{Glu} under conditions where the modified species is only partially charged is thus the only consequence of the faster rate of aminoacylation of intact tRNA^{Glu}.

Significance of the Biphasicity of the BrCN-Treated tRNA^{Glu} Aminoacylation Kinetics. We have already discussed that the modified tRNA^{Glu} preparations used were homogeneous as a prerequisite for this work. This was supported by the analysis of the aminoacylation plateaus. Other arguments prove that the biphasic kinetics are not linked to the presence of contaminating intact tRNA^{Glu} in the BrCN-treated tRNA^{Glu} preparation which would be charged faster than the modified tRNA^{Glu} would be: (1) in the presence of a constant enzyme concentration, the bursts of the Glu-tRNA^{Glu} synthesis are a function of, without being proportional to, the modified tRNA^{Glu} concentration, until saturation is reached; (2) in the presence of a saturating concentration of modified tRNA^{Glu}, the bursts of Glu-tRNA^{Glu} synthesis are proportional to the enzyme concentration; (3) the experimental burst values of the synthesis of modified Glu-tRNA^{Glu} are very similar to the theoretical values determined assuming that they result from the existence of a rate-determining step succeeding the catalytic step of Glu-tRNA^{Glu} synthesis (see the Appendix). The steady state corresponds to the establishment of an equilibrium between the fast rate of synthesis of modified Glu-tRNA^{Glu} (measured during the first catalytic cycle of the enzyme) and a slow step succeeding it. Thus, the steady-state rate of modified Glu-tRNA^{Glu} formation corresponds to that of regeneration of the enzyme form able to initiate a new catalytic cycle.

The aminoacylation kinetics of intact tRNA^{Glu} did not show any variation in the rate of aminoacylation during the first catalytic cycle. Thus, the rate-determining step in this aminoacylation process is either the catalytic step of Glu-tRNA^{Glu} synthesis or another one preceding it. As it is generally accepted that the association of substrates with enzymes is diffusion-controlled (Holler, 1978), this rate is probably that of synthesis of intact Glu-tRNA^{Glu}.

The rate-determining step in the modified tRNA^{Glu} charging reaction could a priori be the dissociation of one of the end products, modified Glu-tRNA^{Glu}, AMP, or PP_i, especially modified Glu-tRNA^{Glu} because it is the end product with the highest affinity for the synthetase. This last alternative has already been proposed to interpret similar results in the *E. coli* isoleucylation and the yeast valylation systems (Eldred & Schimmel, 1972; Fersht et al., 1978).

E. coli glutamyl-tRNA synthetase catalyzes the tRNA-dependent [^{32}P]PP_i–ATP isotope exchange at a significantly faster rate than the formation of Glu-tRNA^{Glu} at the steady state, even after modification of tRNA^{Glu}, indicating that the dissociation of PP_i cannot be rate determining for the overall aminoacylation process (Table I). In addition, it also seems difficult to explain these results by a decrease in the rate of dissociation of AMP, since (1) steady-state kinetic measurements in the nonmodified system showed that AMP and PP_i dissociate randomly from the enzyme·end products complex (Kern and Lapointe, unpublished experiments), and (2) the dissociation of PP_i is not rate determining. However, as suggested for other aminoacylation systems (Eldred & Schimmel, 1972; Fersht et al., 1978), a slow dissociation of the enzyme·Glu-tRNA^{Glu} complex could account for the rate decrease after the first turnover of the enzyme:

$$\text{E·Glu-tRNA}^{Glu}\text{·AMP·PP}_i \xrightarrow{fast} \text{E·Glu-tRNA}^{Glu} + \text{AMP} + \text{PP}_i$$

$$\xrightarrow{slow} \text{E} + \text{Glu-tRNA}^{Glu}$$

Nevertheless, the biphasic kinetics could also reflect the existence of a transconformation step either preceding this dissociation

$$\text{E·tRNA}^{Glu}\text{·ATP·Glu} \xrightarrow{fast} \text{E*·Glu-tRNA}^{Glu}\text{·AMP·PP}_i$$

$$\xrightarrow{fast} \text{E*·Glu-tRNA}^{Glu} + \text{AMP} + \text{PP}_i$$

$$\xrightarrow{slow} \text{E·Glu-tRNA}^{Glu}$$

$$\xrightarrow{fast} \text{E} + \text{Glu-tRNA}^{Glu}$$

or following it

$$\text{E*·Glu-tRNA}^{Glu}\text{·AMP·PP}_i \xrightarrow{fast} \text{E*} + \text{Glu-tRNA}^{Glu} + \text{AMP} + \text{PP}_i$$

$$\xrightarrow{slow} \text{E}$$

In both mechanisms, the rate-determining step would be a slow conformational change E* → E preceding the initiation of a new catalytic cycle. Finally, these results could also be explained by the reaction scheme

first catalytic cycle

$$\text{E} + \text{tRNA}^{Glu} + \text{ATP} + \text{Glu} \xrightarrow{k_a} \text{E*} + \text{Glu-tRNA}^{Glu} + \text{AMP} + \text{PP}_i$$

subsequent catalytic cycles

$$\text{E*} + \text{tRNA}^{Glu} + \text{ATP} + \text{Glu} \xrightarrow{k_a'} \text{E*} + \text{Glu-tRNA}^{Glu} + \text{AMP} + \text{PP}_i$$

where the enzyme would irreversibly transconform (E → E*) during the first catalytic cycle of charging of the modified tRNA^{Glu} and this modification would result in a decrease of the rate constant of catalysis of the further cycles ($k_a' < k_a$).

We discuss now whether the dissociation of the enzyme·modified Glu-tRNA^{Glu} complex or any transconformation is the rate-determining step. The kinetic studies at the pre steady

Scheme I: Pathway of $tRNA^{Glu}$ Glutamylation Catalyzed by Glutamyl-tRNA Synthetase

$$E + ATP + aa + tRNA \overset{k_s}{\rightleftharpoons} \underset{A}{(E, ATP, aa, tRNA)} \overset{k_1}{\rightleftharpoons}$$

$$\underset{B}{(E, aa\sim AMP, tRNA)}\ PP_i \overset{k_2}{\rightleftharpoons} \underset{C}{(E, aa\text{-}tRNA, AMP)} \overset{k_3}{\longrightarrow}$$

$$E + aa\text{-}tRNA + AMP$$

state reported here show that modification of $tRNA^{Glu}$ results in an about 40-fold increase of the K_m value of $tRNA^{Glu}$ for the synthetase and that the K_m in the $[^{32}P]PP_i$–ATP isotope exchange increases similarly. As in the latter reaction, $tRNA^{Glu}$ acts only as an activator, and the K_m represents the dissociation constant of this system. On the other hand, a $K_m(tRNA^{Glu})/K_i(Glu\text{-}tRNA^{Glu})$ ratio equal to 1 has been determined in the modified aminoacylation system (results not shown). All these results indicate a decrease of the affinity of the synthetase for $tRNA^{Glu}$ (and $Glu\text{-}tRNA^{Glu}$) after the modification. Therefore, a slower dissociation of the modified $Glu\text{-}tRNA^{Glu}$ (as compared to the intact $Glu\text{-}tRNA^{Glu}$), resulting from an increase of its affinity for the enzyme, cannot be invoked. It appears thus that the rate-determining step is rather a transconformation involving either the complex of enzyme and modified $Glu\text{-}tRNA^{Glu}$ or the free enzyme. By use of modified substrates, decreases in the rates of catalysis after a first catalytic cycle were described for some enzymes qualified as "mnemonic" by Ricard et al. (1974) and Meunier et al. (1974). Such putative mnemonic modification of glutamyl-tRNA synthetase especially induced by the modified $tRNA^{Glu}$ would, however, affect only the region of the catalytic center responsible for the synthesis of $Glu\text{-}tRNA^{Glu}$ but not that catalyzing the $[^{32}P]PP_i$–ATP exchange, since this reaction occurs at the same rate in the presence of either intact or modified $tRNA^{Glu}$.

$tRNA^{Glu}$ as an Activator of the $[^{32}P]PP_i$–ATP Isotope Exchange. Glutamyl-tRNA synthetase belongs to the family of synthetases requiring their cognate tRNA to catalyze the isotope exchange (Mehler & Mitra, 1967; Folk, 1971; Lapointe & Söll, 1972). This kinetic property was used as a major argument for the existence of a concerted mechanism for these enzymes (Loftfield & Eigner, 1969). However, the present study offers two arguments supporting a two-step aminoacylation pathway for glutamyl-tRNA synthetase. (1) The maximal rates of synthesis of both intact and modified $Glu\text{-}tRNA^{Glu}$ are much slower than the rate of incorporation of $[^{32}P]PP_i$ into ATP. As $Glu\text{-}tRNA^{Glu}$ cannot stimulate the $[^{32}P]PP_i$–ATP exchange faster than it is synthesized, it cannot be an obligatory intermediate for the isotope exchange. (2) Modification of $tRNA^{Glu}$ by BrCN affects the rate of $tRNA^{Glu}$ charging without affecting the rate of isotope exchange, indicating also that the catalytic step of $tRNA^{Glu}$ charging is not involved in the isotope exchange. Therefore, the enzyme must be activated by $tRNA^{Glu}$ to catalyze the isotope exchange that occurs at the equilibrium of the first step of the overall aminoacylation process and that consists in the activation of the amino acid (Scheme I).

As $Glu\text{-}tRNA^{Glu}$ acts as a competitive inhibitor respective to $tRNA^{Glu}$ in the isotope exchange, one would expect a decrease in the rate of this reaction in the presence of modified $tRNA^{Glu}$ since $Glu\text{-}tRNA^{Glu}$ dissociates more slowly from the enzyme than the isotope exchange occurs. As linear incorporations of $[^{32}P]PP_i$ into ATP were observed during the incubation times, it appears that the rate measurements of isotope exchange were effected at the pre steady state of the aminoacylation reaction or that the PP_i present sufficiently reduced the rate of the transfer step to prevent the accumulation of the complex of enzyme and modified $Glu\text{-}tRNA^{Glu}$.

Regions of $tRNA^{Glu}$ Involved in the Amino Acid Activation and in the Transfer Steps. We showed that the integrity of the 3′-OH end of $tRNA^{Glu}$ is required for the stimulation of the amino acid activation step, since periodate-oxidized $tRNA^{Glu}$ and $Glu\text{-}tRNA^{Glu}$ cannot promote the isotope exchange (Kern & Lapointe, 1979). Consequently, this region of $tRNA^{Glu}$ is involved in the amino acid activation step. However, other modifications in the structure of $tRNA^{Glu}$ can maintain this reaction unaffected. As reported here, this is the case when the thiolated uridine derivative in the anticodon is modified by BrCN. Interestingly enough, this modification decreases (1) the affinity of $tRNA^{Glu}$ for the synthetase, (2) the rate of transfer of the activated glutamate to $tRNA^{Glu}$, and (3) another step following the transfer step and which becomes rate determining for the overall glutamylation process. So, the anticodon region of $tRNA^{Glu}$ is not involved in the amino acid activation step. However, it plays an important role in the factors determining the kinetic parameters involved in the interaction of the tRNA with the synthetase as well as in some other partial steps, in particular in the transfer of amino acid to tRNA and in another partial reaction following it.

Conclusion

Chemical modifications of tRNAs have often been used as a probe to study the sites of interaction with their cognate aminoacyl-tRNA synthetases [e.g., Goddard (1977) and Ofengand (1977)]. We have shown that chemical modifications can also be used to study and even to visualize some partial steps of the overall aminoacylation process. The specific modification of the 5-(methylaminomethyl)-2-thiouridine residue at the 5′ end of the anticodon of *E. coli* $tRNA_2^{Glu}$ by BrCN results in a decrease in the rate of $Glu\text{-}tRNA^{Glu}$ synthesis without affecting the rate of isotope exchange. Until now, it has not been possible, in aminoacylation systems that require their cognate tRNA for catalyzing the $[^{32}P]PP_i$–ATP exchange, to dissociate the two functions (stimulation of the isotope exchange and amino acid acceptance) of tRNA. The chemical modification of $tRNA^{Glu}$ studied here constitutes the first successful attempt of dissociation of these two functions of the tRNA in such systems. In addition, this modification allows the visualization of a partial step which does not appear in the presence of intact $tRNA^{Glu}$: we suggest that it is a transconformation of the enzyme preceding the dissociation of the enzyme-$Glu\text{-}tRNA^{Glu}$ complex.

Appendix

Theoretical Determinations of the Amplitudes of the Bursts of $Glu\text{-}tRNA^{Glu}$ Formation for the Experiments Reported under Results. Glutamyl-tRNA synthetase catalyzes the aminoacylation of $tRNA^{Glu}$ via a two-step pathway (see Discussion). This reaction can be represented in a simplified form according to Scheme I: A, B, and C are various intermediates which are formed during the overall glutamylation process, and E represents the free enzyme form; k_s, k_1, k_2, and k_3 are the rate constants of the various steps.

When intact $tRNA^{Glu}$ is aminoacylated, no decrease in the rate of $Glu\text{-}tRNA^{Glu}$ formation occurs after the first catalytic cycle. As the isotope exchange occurs faster than the aminoacylation, it can be assumed that the rate-determining step corresponds to the transfer of the activated amino acid to tRNA ($k_1 > k_2$ and $k_3 \geq k_2$). However, when BrCN-treated $tRNA^{Glu}$ is aminoacylated, the rate-determining step takes place between the transfer step (B → C) and the initiation of the next catalytic cycle (A → B), indicating that $k_3 < k_2$. This

Table II: Apparent First-Order Rate Constants of the Various Steps of Aminoacylation of BrCN-Treated tRNA^{Glu} and Comparison of the Theoretical and Experimental Burst Values of Modified Glu-tRNA^{Glu} Formation[a]

			step (see Scheme I)			burst values (μM Glu-tRNA^{Glu})	
experiment	tRNA (μM)	enzyme (μM)	A → B k_1' (s^{-1})	B → C k_2' ($10^2\ s^{-1}$)	C → E k_3' ($10^2\ s^{-1}$)	theoretical	experimental
1	50.0	0.05	0.96	0.598	0.180	0.53	0.52
2	50.0	0.11	0.96	0.598	0.179	1.07	1.04
3	50.0	0.22	0.96	0.608	0.182	2.14	2.20
4	50.0	0.44	0.96	0.584	0.183	4.28	4.40
5	1.0	0.22	0.34	0.154	0.096	0.61	0.59
6	1.5	0.22	0.44	0.203	0.113	0.84	0.85
7	3.0	0.22	0.60	0.323	0.144	1.35	1.33
8	5.0	0.22	0.71	0.393	0.156	1.59	1.58
9	10.0	0.22	0.81	0.491	0.170	1.88	1.85
10	50.0	0.22	0.96	0.617	0.183	2.17	2.22
11	100.0	0.22	0.96	0.617	0.183	2.17	2.22

[a] The theoretical calculations are in the Appendix. Experiments 1-4 and 5-11 correspond to those presented, respectively, in Figures 2 and 3.

last kinetic behavior is like that described by Fersht (1978) for chymotrypsin reacting with (*p*-nitrophenyl)acetate and by Fersht et al. (1978) for the yeast arginylation system. These authors demonstrated that, provided $k_s >> k_1$, k_2, and k_3, the amplitude I of the burst of synthesis of the end product dissociating the last is given by

$$I = [E]_0 \frac{(k_1'k_2')^2}{(k_1'k_2' + k_2'k_3 + k_3k_1')^2}\left(1 - \frac{k_3^2}{k_1'k_2'}\right) \quad (2)$$

where $[E]_0$ is the concentration of enzyme and k_1' and k_2' are the apparent first-order rate constants of the corresponding steps (respectively A → B and B → C in Scheme I), which follow, in general, the Michaelis–Menten equation. Thus, at saturating substrate concentrations, k_1' and k_2' can be replaced in eq 2 by their absolute rate constants k_1 and k_2.

The apparent rate constant k_{exch} of the [^{32}P]PP$_i$–ATP exchange corresponds to a minimal value of the rate constant k_1' of the amino acid activation (Cole & Schimmel, 1970; Holler, 1978); if $k_{exch} >> k_2'$ and k_3, indicating that $k_1' >> k_2'$ and k_3, eq 2 can be simplified as (Fersht, 1978)

$$I = [E]_0\left(\frac{k_2'}{k_2' + k_3}\right)^2 \quad (3)$$

Determinations of the Amplitudes of the Bursts of Modified Glu-tRNA^{Glu} Formation in the Presence of a Saturating Concentration of BrCN-Treated tRNA^{Glu} and Various Concentrations of Glutamyl-tRNA Synthetase (cf. Experiments Shown in Figure 2). Table II shows the experimental values of the apparent first-order rate constants of the various steps of the kinetics presented in Figure 2: k_{exch} (a minimal value of k_1' corresponding to step A → B), k_2' and k_3' (the apparent first-order rate constants of steps B → C and C → E). The absolute rate constant k_3 of step C → E is defined by (Fersht, 1978)

$$k_3'[E]_0 = [E]_0\frac{k_2'k_3}{k_2' + k_3} \quad (4)$$

where $k_3'[E]_0$ represents the slope of the slow linear phase of the reaction. A value of 0.0026 s^{-1} was determined for k_3. As $k_{exch} = 0.96\ s^{-1}$ and $k_2' = 0.60\ s^{-1}$, it follows that $k_1' >> k_2'$ and k_3. Equation 3 can be used for the theoretical determinations of I. Table II shows that these values are in agreement with the experimental burst values.

Determination of the Amplitudes of the Bursts of Modified Glu-tRNA^{Glu} Formation in the Presence of Limiting Concentrations of BrCN-Treated tRNA^{Glu} (cf. Experiments Shown in Figure 3). As tRNA^{Glu} acts as an activator in the first step A → B (cf. Discussion), before use of the simplified eq 3 for the calculations of I, it must be verified that, even in the presence of limiting concentrations of modified tRNA^{Glu}, $k_1' >> k_2'$ and k_3; if not, eq 2 should be used.

The apparent rate constant k_{exch} of the isotope exchange (a minimal value of k_1') follows the Michaelis–Menten equation

$$k_{exch} = \frac{k_{cat}[S]}{K_m + [S]} \quad (5)$$

where k_{cat} represents the rate constant of the isotope exchange in the presence of a saturating concentration of modified tRNA^{Glu}, [S] is the concentration of modified tRNA^{Glu}, and K_m is the Michaelis constant of this system. Table II shows for the various modified tRNA^{Glu} concentrations tested (from 1 to 100 μM) the corresponding k_{exch}, k_2' and k_3' values. k_3 determined from the various slopes of the slow linear phases of the kinetics (Figure 3) is found to be equal to 0.0026 s^{-1}. It appears that for each modified tRNA^{Glu} concentration tested k_{exch} and thus k_1' are much higher than k_2' and k_3. Again the burst values determined according to eq 3 are in agreement with the experimental values (Table II).

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Spin Equilibrium and Quaternary Structure Change in Hemoglobin A. Experiments on a Quantitative Probe of the Stereochemical Mechanism of Hemoglobin Cooperativity†

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ABSTRACT: The molecular mechanism of hemoglobin cooperativity was studied kinetically by flash photolysis on mixed-state hemoglobins which consist of three ferrous carboxy subunits and one hybrid ferric subunit including fluoromet, azidomet, cyanatomet, and thiocyanatomet. The effects of conformational transitions on the hybrid subunit were detected by kinetic absorption spectroscopy after the CO was fully photodissociated from the binding sites by a large pulse of light from a tunable dye laser. The hemoglobin conformational transition rate was observed to depend on its state of ligation. At 22 °C, pH 7, and 0.1 M phosphate, the deoxy R → T conformational change rate is 4×10^4 s^{-1}. The rate decreases to 1.4×10^4 s^{-1} for singly ligated hemoglobin. The R → T conformation change alters the energy separation between the high- and low-spin states for azidomet, cyanatomet, and thiocyanatomet subunits by about 700, 300, and 300 cal/mol, respectively. There are two possible implications of this result: (1) the iron atom spin state is not the only major factor in the determination of its position with respect to the heme plane or (2) the change with conformation of the protein force exerted by the proximal histidine on the iron atom (for an iron to heme-plane displacement of less than 0.3 Å) is less than 50% of that expected from simple models in which this motion is responsible for cooperativity.

The essence of the cooperative binding of oxygen by mammalian hemoglobin lies in the several 100-fold difference in the oxygen affinity between the two quaternary structures of the hemoglobin molecule. While there are measurable structural changes within one particular subunit when a second subunit is ligated, the energy of binding at a given subunit depends chiefly on the quaternary structure (and pH, temperature, and other aspects of solution conditions), not on the state of ligation per se of the other subunits (Shulman et al., 1975; Perutz, 1976). One thus seeks a quantitative structural understanding of the way in which the binding energy of oxygen (or other ligands) can affect and be affected by the quaternary structure of the protein. The problem is made difficult by the fact that many of the observed structural changes do not have a significance for the oxygen affinity, by the fact that only a small amount of the affinity change is localized as an energy at the heme (Ogawa & Shulman, 1972; Susser et al., 1974), and by the myriad of small structural changes observed.

The most conspicuous heme structural change occurring upon oxygenation is the movement of the iron atom from 0.6 Å out of the mean porphyrin plane into coplanarity with the heme (Perutz, 1970; Fermi, 1975). Simultaneously, the iron changes from a high-spin to low-spin state. The conventional explanation for such a 0.6-Å geometrical difference, according to Hoard (1971), is that the high-spin deoxy iron atom has too large an effective ionic radius to be fitted into the porphyrin core. However, the out-of-plane nature may also arise from steric repulsions between the axial ligands and the porphyrin nitrogen orbitals (Olafson & Goddard, 1977; Warshel, 1977). Hoard (1971) and Perutz (1970) have both proposed that the iron–proximal histidine coordinate is central to the cooperativity and that the δ_L movement of 0.6 Å acts as a trigger for the concerted movement of the protein framework. The more recent model compound studies (Kastner et al., 1978) combined with EXAFS studies (Eisenberger et al., 1979) put into question the simple connection between spin, ionic radius, and

† From the Department of Physics, Princeton University, Princeton, New Jersey 08540. *Received December 12, 1978; revised manuscript received May 31, 1979.* This work was supported in part by Grants DMR-75-142C4 and DMR-78-05916.

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0006-2960/79/0418-5826$01.00/0