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Interactions between *Escherichia coli* Arginyl-tRNA Synthetase and Its Substrates†

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ABSTRACT: Interactions between *Escherichia coli* arginyl-tRNA synthetase and its substrates were extensively studied and distinctly demonstrated. Various approaches such as equilibrium dialysis, fluorescence titration, and substrate protection against heat inactivation of the enzyme were used for these studies. In the absence of other substrates, the equilibrium dissociation constants for arginine, ATP, and the cognate tRNA were about 70 μM, 0.85 mM, and 0.45 μM, respectively, at pH 7.5, in Tris buffer. The binding of arginine to the enzyme was affected neither by the presence of tRNA nor by the presence of ATP but was considerably enhanced when ATP and tRNA were both present at saturating concentrations. The dissociation constant in this case (about 16 μM) was very close to the K_m (12 μM) for arginine during aminoacylation. The binding of ATP (the equilibrium dissociation constant $K_D \approx 0.85$ mM) was not affected by the presence of arginine but was depressed in the presence of tRNA (K_D became 3 mM). Arginyl-tRNA showed a dissociation constant of (4–5) $\times 10^{-7}$ M which was not affected by the presence of a single other substrate. Possible explanations for the high K_m for tRNA in the aminoacylation are discussed. Our results indicated pronounced interactions between substrates mediated by the enzyme under catalytic conditions. Periodate oxidation did not alter the tRNA binding to the enzyme. The oxidized tRNA still afforded protection against heat inactivation of the enzyme.

The mechanism of a small group of aminoacyl-tRNA synthetases represented by arginyl-tRNA synthetase (and glutamyl- and glutaminyl-tRNA synthetases) has long been discussed [e.g., Mitra and Mehler (1967), Craine and Peterkofsky, (1975), Fersht et al. (1978), Charlier and Gerlo (1979), Thiebe (1983), and Char and Gopinathan (1986)]. Numerous studies have been devoted to the interactions between the synthetases and their substrates.

For example, for the arginyl-tRNA synthetase, Mitra et al. (1970) reported that arginine and tRNA protected the *Escherichia coli* B enzyme against heat inactivation but ATP did not (pH 5.5, Mes buffer). Arginine and tRNA showed a "synergistic protection" (pH 8, Tris, or pH 5.5, Mes).[1] Differently, Charlier and Gerlo (1979) reported that for the *E. coli* K12 enzyme no influence was observed between the binding of arginine and the binding of other substrates whereas the presence of ATP decreased the binding of tRNA and vice versa through steady-state kinetics with initial velocity and inhibition studies (pH 7.4, Hepes).

Parfait and Grosjean (1972) reported for the enzyme from *Bacillus stearothermophilus* that arginine was bound only after the binding of tRNA and ATP; the addition of substrates is ordered sequential (pH 7.4, Hepes). Nazario and Evens (1974) also reported the ordered sequential mechanism in *Neurospora crassa*, but more reports with *E. coli* B (Papas

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[1] Abbreviations: Arg, arginine; ArgRS, arginyl-tRNA synthetase; $tRNA^{Arg}$, arginine-specific tRNA; DTT, dithiothreitol; $K_{S_1}^{S_2}$, equilibrium dissociation constant of substrate S_1 in the presence of S_2; $K_{S_1}^{S_2S_3}$, dissociation constant of substrate S_1 in the copresence of S_2 and S_3; Mes, 2-(*N*-morpholino)ethanesulfonic acid; S_1–S_3, any of the three substrates—arginine, ATP, and $tRNA^{Arg}$; Tris, tris(hydroxymethyl)aminomethane; $tRNA_{ox}^{Arg}$, $tRNA^{Arg}$ of which the 3′-terminal adenosine is oxidized by periodate.

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& Peterkofsky, 1972), bakers' yeast (Freist et al., 1981), human placenta (Wang & Pan, 1984), etc. showed a random sequential mechanism for arginyl-tRNA synthetase.

The lack of similarity in published results on substrate interactions of arginyl-tRNA synthetases might be suggested to reflect the different enzyme sources or different experimental conditions employed. However, contradictory results have been reported even for the enzyme from a single origin. For example, for the arginyl-tRNA synthetase from *B. stearothermophilus*, Pafait and Grosjean (1972) showed an ordered sequential mechanism for the substrate addition which led to the conclusion of a concerted mechanism for the aminoacylation (pH 7.4, Hepes buffer). On the other hand, Godeau (1980) reported a ping-pong mechanism which indicated that the aminoacylation took place via the aminoacyl adenylate intermediate (pH 7.4, Hepes buffer).

Until now, quantitative results with stepwise demonstration of each of the arginyl-tRNA synthetase–substrate interactions were seldom reported. We have undertaken extensive studies of these interactions in order to show the influence of one or more substrates on the binding of another.

For the *E. coli* K12 arginyl-tRNA synthetase, Charlier and Gerlo (1979) showed the substrate interactions mentioned above and also a random addition of substrates by steady-state kinetics at pH 7.4, Hepes buffer. Here we report the distinct demonstration of the enzyme–substrate interactions. Different approaches were used to study each specific binding in order to confirm the results.

Materials and Methods

Materials

Arginyl-tRNA synthetase (ArgRS) of *E. coli* K12 was purified to more than 95% homogeneity as reported in the preceding paper (Lin et al., 1988). Pure *E. coli* $tRNA^{Arg}$ with a charging capacity of more than 1400 pmol/A_{260} unit was purchased from Subriden RNA Co., Rollingbay, WA. L-Arginine and ATP were from Sigma (St. Louis, MO). Uniformly labeled L-[^{14}C]arginine (300–400 mCi/mmol) was obtained from Amersham (England).

Methods

Unless otherwide stated all experiments were carried out in a standard buffer containing 50 mM Tris-HCl (pH 7.5), 3 mM excess Mg^{2+}, 80 mM KCl, and 0.2 mM DTT.

Equilibrium Dialysis. Experiments were performed according to Furlong et al. (1972) in eight lucite cells (each chamber of the cells has a volume of 100 μL, and the two chambers of the cells were separated by cellulose membranes cut from visking dialysis tubes). Each compartment was filled with 50 μL of the enzyme or ligand solution in the standard buffer containing the same concentration of glycerol (10–20%). One chamber of each cell was filled with ArgRS (9–10 μM) and the other with [^{14}C]arginine (10–300 μM). In the examination of the influence of $tRNA^{Arg}$ on the binding of arginine, a saturating concentration (12 μM) of $tRNA^{Arg}$ was contained on the enzyme side. The dialysis was run for 5–8 h at 4 °C while the cells were slowly rotated. Samples of 30 μL or duplicates of 15 μL were withdrawn from each chamber with the microsyringe and counted in 0.6% butyl-PDB [2-(4-*tert*-butylphenyl)-5-biphenyl-4-yl-1,3,4-oxadiazole] in toluene/ethylene glycol monomethyl ether (volume ratio 4:6). Nearly no decrease of enzyme activity could be found in the experiment under our condition.

Fluorescence Titration. The association of ArgRS and its substrates was monitored by the change in the intrinsic fluorescence intensity of the enzyme. Aliquots (0.1–2 μL) of ligand solutions were added to an enzyme solution of 0.22 mL in the microcell of a Hitachi MPF-4 fluorometer. The enzyme solution was in the standard buffer which mimicked the buffer system of the aminoacylation (including protecting reagents, see Materials and Methods of the preceding paper). Excitation and emission wavelengths were 295 and 335 nm with bandwidths of 1.75 and 18 nm, respectively. Substrate–substrate interactions can be monitored by titration with one substrate in the presence of saturating amounts of another. The enzyme consumption is as low as 2–3 μg for most experiments.

In the titration experiments with ATP–Mg^{2+} (it was considered as the true substrate, refer to the preceding paper) or arginine, excess substrate concentrations were used ([S] ≫ $[E]_t$ = 0.14–0.3 μM, $[E]_t$ denotes the input enzyme concentration). The affinity of these substrates was not high (as compared with tRNA) and a simplified linear relation could be derived:

$$\Delta F = -K_D \Delta F/[S] + \Delta F_\infty \quad (1)$$

where ΔF, ΔF_∞, and K_D refer to the fluorescence change at the substrate concentration [S], the fluorescence change when all enzyme molecules are complexed with substrates, and the dissociation constant of the enzyme–substrate complex, respectively (Holler et al., 1971).

Titrations with $tRNA^{Arg}$ were started from [tRNA] = [S] < $[E]_t$, and an input enzyme concentration $[E]_t$ approaching the estimated dissociation constant was used to give detectable free and bound ligands. Scatchard plots (Segel, 1975) were used for the data analysis. As ATP and tRNA both absorb at the excitation wavelength (295 nm), in our experiments the absorption of samples was followed throughout the titration for accurate inner-filter corrections. A small excitation slit (1.75 nm) was chosen to minimize photodecomposition of the enzyme over the time range of 30 or 60 min required for an experiment while the big emission slit was adjusted to provide an adequate signal to noise ratio.

Data Processing. Interactive programs based on a least-squares method were designed for both equilibrium dialysis (using a Scatchard plot) and fluorescence titration (calculations based on eq 1 or based on a Scatchard plot with tRNA titration). An Apple II or IBM-PC microcomputer was used. The original experimental data were entered, then both volume and inner-filter corrections (when necessary) were made with the programs, and final linear relations were given out with calculated K_D, n (the number of binding sites of the substrate to the enzyme), and corresponding graphs.

The inner-filter correction was made according to Burstein (1968):

$$f = \frac{I_0}{I} = \frac{P_0 + \Delta A}{P_0} \frac{1 - 10^{-P_0}}{1 - 10^{-(P_0+\Delta A)}}$$

where f, I_0, I are the correction factor and the corrected and observed fluorescence intensity, respectively. P_0 and ΔA denote the sample absorption before titration and the absorption change with addition of substrates.

Heat Inactivation. The conditions of heat inactivation mimicked in all respects those of aminoacylation except that the inactivation was conducted at 55 °C and all substrates were not present simultaneously. The incubation mixtures contained catalytic amounts of ArgRS (15 μg/mL) in the standard buffer (adjusted to be at pH 7.5 at 55 °C) with 0.4 mg/mL BSA and 5% glycerol. Samples of 40 μL without substrates or with substrates in different concentrations were equilibrated at 0 °C and then at room temperature for several minutes before inactivation. After the samples had been heated for 5 min at 55 °C, they were immediately diluted (about 50–

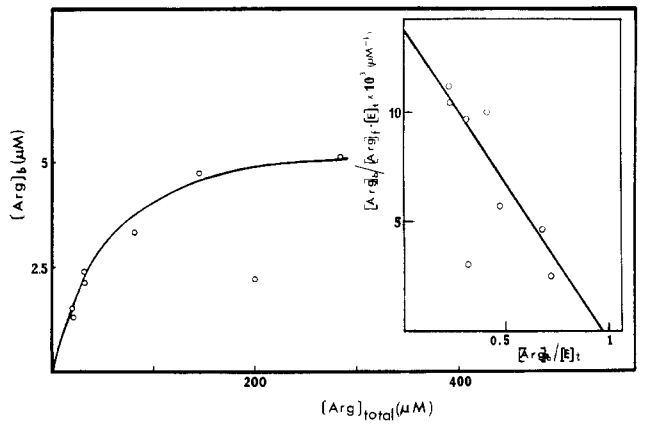

FIGURE 1: Equilibrium dialysis between arginine and ArgRS. The experiments was carried out with 9–10 μM ArgRS and 10–300 μM [^{14}C]arginine (10 μCi/μmol) in the standard buffer for 5–8 h at 4 °C. In the insert is the corresponding Scatchard plot of the dialysis. $[Arg]_b$, $[Arg]_f$, and $[E]_t$ refer to the bound arginine, free arginine, and input enzyme concentrations, respectively.

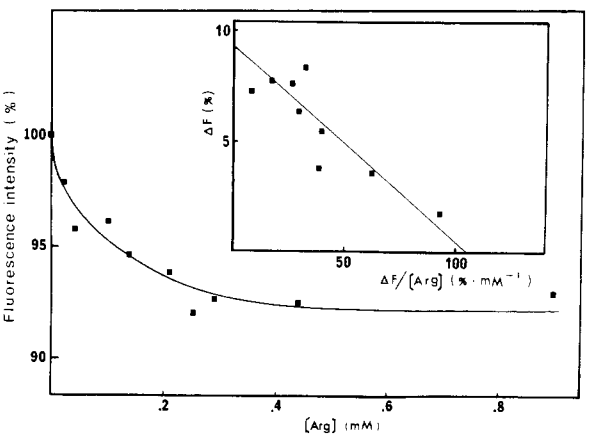

FIGURE 2: Titration of the ArgRS fluorescence by arginine. Conditions are given under Materials and Methods. In the insert is the corresponding Eadie plot from which the slope indicated a K_{Arg} value of about 75 μM.

80-fold) and cooled in the buffer for enzyme dilution (see Materials and Methods of the preceding paper) preequilibrated in ice. The remaining enzyme activities were determined by measuring the initial velocity of the aminoacylation reaction.

Before the study of substrate-induced protection of enzyme, it was determined that the inactivation rate of enzyme or enzyme in the presence of the substrates of interest followed first-order kinetics (verified by measuring the remaining enzyme activities after various time intervals ranging from 1 to 10 min). The inactivation time, 5 min, was selected so that, in the absence of substrates, about 25% of the initial enzyme activity remained at the end of the inactivation period.

Preparation of $tRNA^{Arg}_{ox}$. The oxidation of the 3′-terminal adenosine of $tRNA^{Arg}$ (100 μM) was carried out in the presence of 1 mM freshly prepared periodate containing 10 mM Mg^{2+} at pH 4.5 and 0 °C, then the reaction was stopped with the addition of glycerol (10% v/v final concentration), and the modified tRNA was dialyzed against bidistilled water and concentrated by ethanol precipitation similar to that in Lin et al. (1984). No tRNA charging capacity could be found after this modification.

RESULTS

Interactions of Arginine with ArgRS and Other Substrates

In the Absence of Other Substrates. The binding of arginine was studied by equilibrium dialysis. The Scatchard plot revealed a K_{Arg} = 70 ± 15 μM and the number of binding sites = 1 (0.95 is shown in the plot, insert of Figure 1). The average value of several fluorescence titrations carried out from 2 to 1000 μM arginine also gave a K_{Arg} = 75 ± 15 μM (Figure 2) with a maximum fluorescence quenching of about 9% of the intrinsic enzyme fluorescence (by data plotting, Figure 2), thus confirming the results of the equilibrium dialysis.

In the Presence of Other Substrates. The fluorescence titration by arginine in the presence of a saturating concentration of ATP (15 mM) gave an enhancement of the enzyme fluorescence of 15–20%, but no alteration of the dissociation constant of arginine could be detected (Table I).

Both equilibrium dialysis and fluorescence titration in the presence of a saturating concentration of $tRNA^{Arg}$ (12 and 3 μM in the two experiments, respectively) indicated that the binding constant of arginine was not altered under this condition (Table I). The equilibrium dialysis in the presence of tRNA revealed also one binding site for arginine. The relative low concentration of 3 μM tRNA was used in the fluorescence study to avoid a large inner-filter effect of the tRNA on the initial fluorescence intensity, while still maintaining saturation. A maximum fluorescence increase of about 12–14% with arginine was found under this condition.

Table I: Substrate Binding of Arginyl-tRNA Synthetase[a]

	additions[b]	fluorescence titration	equilibrium dialysis	K_m in aminoacylation[c]
K_{Arg} (μM)	none	75 ± 10	70 ± 15	
	+ATP	75 ± 10		
	+$tRNA^{Arg}$	80 ± 10	70 ± 15	12
	+ATP + $tRNA^{Arg}$	16 ± 4		
K_{ATP} (mM)	none	0.85 ± 0.2	1.5 ± 0.3[d]	
	+Arg	0.9 ± 0.2		0.9
	+$tRNA^{Arg}$	3 ± 0.5	3 ± 0.6[d]	
K_{tRNA} (μM)	none	0.45 ± 0.1		
	+Arg	0.5 ± 0.1		2.5
	+ATP	0.45 ± 0.1		

[a] Experimental conditions mentioned in the text. [b] In the test of binding constants in the presence of other substrates, the latter were presented in saturating concentrations; see the text. [c] For details, refer to preceding paper. [d] Protection against enzyme inactivation.

In the presence of both excess ATP and $tRNA^{Arg}$ (30 mM and 8 μM, respectively), the fluorescence titration with arginine ([Arg] ≫ $[E]_t$) revealed a dissociation constant of $K^{ATP,tRNA}_{Arg}$ = 16 ± 4 μM that was lower than that in the presence of all other combinations of substrates. The fluorescence increase in this case was about 12–14%. The K_D was very similar to the K_m for arginine (12 μM) in the aminoacylation where both ATP and tRNA were present (see preceding paper and Discussion in this paper).

Interactions of ATP with ArgRS and Other Substrates

These interactions were studied by fluorescence titration and verified by enzyme protection against heat inactivation in the presence of different substrate combinations.

Heat Inactivation. First of all, it was verified that the inactivation of ArgRS alone or in the presence of $tRNA^{Arg}$ or ATP or both followed first-order kinetics, tested in the time range of 1–10 min. Both $tRNA^{Arg}$ (10 μM) and ATP (10 mM) induced enzyme protection against heat inactivation; the addition of ATP gave considerably more protection than tRNA alone (data not shown), providing a satisfactory signal for the study of ATP binding in the presence of tRNA.

We chose 5 min for the heat inactivation process in the presence of various amounts of substrates. Data were treated

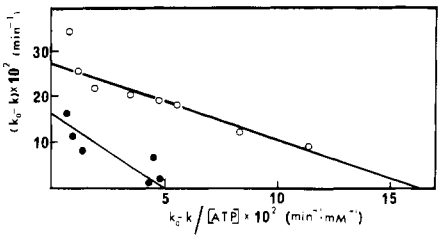

FIGURE 3: Eadie–Hofstee plot of substrate protection against heat inactivation of ArgRS. The slope denotes the equilibrium dissociation constant. The incubation mixture contained 15 μg/mL ArgRS in the standard buffer and was heated at 55 °C for 5 min. (○) In the absence of tRNA; (●) in the presence of a saturating amount of tRNA (10 μM). k_0 and k are respectively the first-order rate constant of inactivation in the absence and in the presence of added ATP. They were calculated as described under Results.

according to Chuang and Bell (1972) with the Eadie–Hofstee-type plot:

$$k_0 - k = -\frac{k_0 - k}{[S]}K_D + (k_0 - k_\infty) \qquad (2)$$

where k_0 and k are respectively the first-order rate constant of the inactivation in the absence and in the presence of added substrtes, whereas k_∞ is the k with saturating substrate. The constants were calculated according to

$$\log (\text{percentage of remaining activity}) = -\frac{k}{2.3}t + 2$$

where t = 5 min.

In studying the binding constant of ATP in the presence of tRNA, k_0 was considered as the inactivation constant of the E–tRNA complex form (instead of E alone) where the process

$$\begin{array}{ccc} (\text{E–tRNA}) + \text{ATP} & \rightleftharpoons & \text{E}:^{\text{tRNA}}_{\text{ATP}} \\ {\scriptstyle k_0}\downarrow & & \downarrow{\scriptstyle k} \\ \text{E}_d\text{–tRNA} & & \text{E}_d:^{\text{tRNA}}_{\text{ATP}} \end{array}$$

was taken into account, thus permitting the evaluation of such dissociation constants (E_d refers to the inactivated form of the enzyme).

ATP Binding in the Absence of Other Substrates. A K_{ATP} value of 0.85 ± 0.2 mM (average of five experiments) was derived from the titrations of the enzyme fluorescence by ATP, with a maximum quenching of about 16 ± 4%. The protection afforded by different concentrations of ATP against heat inactivation also permitted the derivation of K_{ATP}. The average value was 1.5 ± 0.5 mM at 55 °C.

ATP Binding in the Presence of Other Substrates. K_{ATP} was not altered in the presence of arginine as monitored by the fluorescence titration, nor was the percentage of fluorescence quenching (Table I).

Fluorescence titrations with ATP in the presence of saturating $tRNA^{Arg}$ (about 10 μM) gave K_{ATP}^{tRNA} = 3.0 ± 0.5 mM, indicating that the presence of tRNA decreased the affinity of ATP for the enzyme. This result was also verified by the enzyme protection of ATP against heat inactivation in the presence of $tRNA^{Arg}$ with which an average K_{ATP}^{tRNA} = 3 ± 0.6 mM from several experiments was found. This value was also greater than the K_{ATP} in the absence of tRNA found by the heat inactivation study (Figure 3).

Interactions of $tRNA^{Arg}$ with ArgRS and Other Substrates

The $tRNA^{Arg}$ has an affinity much greater than those of arginine and ATP (see preliminary experiment below), so the relation (eq 1) for fluorescence titration is no longer applicable

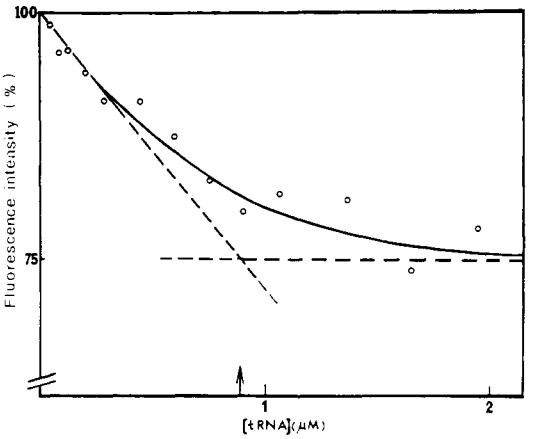

FIGURE 4: Determination of K_{tRNA} with fluorescence titration. The enzyme concentration was 0.3 μM, and other conditions were described under Materials and Methods. The intersection between the initial tangent of the F vs [tRNA] curve and its asymptote gave an abscissa value equal to $n[E]_t + K_D$ (0.88 μM in this experiment, indicated by the arrow); $[E]_t$ and n were the input concentration and number of binding sites of the enzyme, respectively, and they were determined as described under Results.

in this case, as the condition $[S] \gg [E]_t$ could not be satisfied. To obtain sufficient experimental points in the equilibrium region, the titrations were started with substrate considerably below the input enzyme concentration, and the data were treated as described as follows.

tRNA Binding in the Absence of Other Substrates. A preliminary titration with $tRNA^{Arg}$ was carried out with a low enzyme concentration (e.g., $[E]_t$ = 0.08 μM) to give an estimation of the K_{tRNA}. We know that the intersection of the tangent of the initial part of the F vs [S] curve and its asymptote can denote the value of $n[E]_t + K_D$ according to Lefevre et al. (1980). In this case, as the $[E]_t$ was far below the $n[E]_t + K_D$ value, an estimation of K_{tRNA} = 0.46 μM was obtained.

K_{tRNA} was determined by the tRNA titration using $[E]_t$ = 0.3 μM being close to the estimated K_{tRNA} (Figure 4). For the first approximation we assumed that n = 1, so the bound substrate and free substrate concentrations could be derived from $S_b = n[E]_t(\Delta F/\Delta F_\infty)$ and $[S]_f = [S] - [S]_b$ (when the enzyme has one or more than one independent and equivalent sites), and a K_D = 0.5 μM was indicated by the corresponding Scatchard plot. From this K_D the $n[E]_t + K_D$ should be 0.83 μM which is very close to the $n[E]_t + K_D$ = 0.88 μM denoted by the F vs [tRNA] curve (Figure 4). So this approximation was justifiable, and both K_D and n for the tRNA binding were obtained (for a more sophisticated evaluation, see below). The average of several experiments gave the K_{tRNA} = 0.45 ± 0.1 μM with a maximum enzyme fluoresence quenching of about 30%. The tRNA binds to the ArgRS with a 1:1 stoichiometry.

In fact, the K_D and n can be evaluated simultaneously by a computer program with which several n's are input and the corresponding K_D's are derived through the Scatchard plots. One pair of n and K_D is chosen which gives the best fit to the $n[E]_t + K_D$ value obtained from the F vs [S] curve (Lin et al., unpublished results). Analysis in this manner gave the same results as above.

tRNA Binding in the Presence of Other Substrates. The presence of arginine did not alter the binding of tRNA as was monitored by fluorescence titration, nor did the presence of ATP give an evident alteration to this binding. The titration of tRNA in the presence of ATP showed a fluorescence enhancement (about 20%) that differed from the fluorescence quenching in the absence of ATP, which may indicate varying

enzyme conformational changes in the two processes.

Under catalytic conditions tRNA did show a very high K_m (2.5 μM, see preceding paper) which was 5.4 times the K_{tRNA} in the absence of other substrates.

Substrate Bindings in Some Special Cases

ATP Binding at pH 6. MES buffer was used instead of Tris-HCl in this study. Titrations with ATP in the range of 0.1–3 mM showed a quenching of fluorescence, followed by an increase of fluorescence in the range of 2–50 mM. The first part showed a K_{ATP} value of 0.9 ± 0.2 mM, nearly the same as that at pH 7.5. This biphasic curve, which was not seen at pH 7.5, was verified by the protection against enzyme denaturation afforded by ATP (at 50 °C in this case); two binding constants (1.5 and 20–30 mM, respectively) were also found. The nature and specificity of the second binding is being further studied.

Binding of Periodate-Oxidized tRNA. Oxidized $tRNA^{Arg}$ showed essentially identical binding properties as native tRNA as monitored by the fluorescence titration or by protection against heat inactivation of enzyme.

Discussion

Our results demonstrate that arginine binds to the enzyme in the absence of other substrates, which differs from the report that the binding of tRNA and ATP is prerequisite for the binding of arginine, i.e., that ArgRS follows an ordered sequential mechanism for *B. stearothermophilus* at pH 7.4, by Parfait (1973a,b). Though there is some similarity for the increase of arginine affinity in the presence of the other substrates simultaneously, in our report a quantitative result of a 4–5-fold decrease for K_{Arg} is demonstrated, while a more extreme situation that arginine is bound only after the binding of the other substrates was stated in their system. We have shown by different approaches that all three substrates did bind in the absence of other substrates, supporting the mechanism of random substrate addition in ArgRS reported for *E. coli* K12 (Charlier & Gerlo, 1979), *E. coli* B (Papas & Peterkofsky, 1972), bakers' yeast (Freist et al., 1981), and human placenta (Wang & Pan, 1984). As the binding of tRNA is not prerequisite for the binding of either arginine or ATP, our results indicate that the requirement of tRNA for ATP–PP_i exchange is not simply due to the requirement of tRNA for the binding of other substrates, similar to the results of Wang and Pan (1984).

Both the substrate addition and the decrease of ATP affinity in the presence of tRNA were similar to the steady-state kinetic results of Charlier and Gerlo (1979) for the *E. coli* K enzyme.

For many aminoacyl-tRNA synthetases studied thus far, the K_m of tRNA in the aminoacylation and its K_D are usually similar, e.g., for yeast phenylalanyl-tRNA synthetase (Fasiolo et al., 1977, 1981; Baltzinger et al., 1983) and for *E. coli* isoleucyl-tRNA synthetase (Baldwin & Berg, 1966; Lam & Schimmel, 1975). The relative large difference of more than 5-fold between these two constants in our system may have two possible explanations: (1) An antisynergism exists for tRNA binding in the presence of both ATP and arginine. That would also be similar to the results of Charlier and Gerlo (1979) for the *E. coli* K12 enzyme. (2) A special mechanism is present in the catalytic reaction as a result of a different intermediate form of the enzyme (Fersht, 1985).

The synergism of arginine binding in the presence of other substrates and the antisynergism of ATP binding in the presence of tRNA (or the possible antisynergism of tRNA binding in the presence of other substrates) indicate that important conformational changes of the synthetase may occur upon aminoacylation leading to its catalytic active form. Consequently, the ATP–PP_i exchange absolutely requires the presence of tRNA. This might be the characteristic mechanism of this special aminoacyl-tRNA synthetase.

The binding studies were carried out under conditions which mimicked the aminoacylation. A small difference in pH (7.5 for binding and 7.4 for aminoacylation), however, was due to the different buffer preparation. We verified various K_m values of aminoacylation at pH 7.5, and no detectable changes were found from those measured at pH 7.4 in the preceding paper.

In this paper, we obtained quantitative binding data from substrate protection of thermal inactivation of the enzyme according to Chuang and Bell (1972) and developed this method for studies of substrate interactions. The binding constants were calculated from a series of protection percentage values in the presence of different substrate concentrations (the "titration") which revealed the tendency of binding and its saturation. The titration is advantageous over such heat inactivation studies when the binding was only judged by the percentage of protection and the substrate interactions were only judged by comparing a single set of such percentage values in the presence of different substrate combinations. For example, in some previous work, if the protection of enzyme activity in the copresence of substrates A and B was greater than the sum of protections in the presence of A and B separately, a synergism of protection was concluded. However, that does not directly indicate synergism of binding because the binding of different substrate combinations can give rise to varying enzyme conformations with different sensitivities to heat inactivation. Thus, the percentage of protection will not always be proportional to the degree of saturation and will not reflect the binding constants directly. For example, Parfait (1973a) found in *B. stearothermophilus* that a synergistic protection of ArgRS existed with arginine and $tRNA^{Arg}$, similar to that of Mitra et al. (1970) (in *E. coli* B) at a high concentration of arginine (>0.1 mM). However, at lower arginine concentration (2 μM), the copresence of arginine and tRNA did not increase enzyme protection, and the synergistic protection now appeared only when ATP was added to the system. In such a case, the titration approach would provide greater insight into the intrinsic mechanism of substrate interactions. Moreover, the protection depends considerably on the inactivation conditions, such as the time range or the inactivation temperature and the concentration range of the substrates, thus affecting the precision and reliability of the data. The predetermination of the appropriate time range for the first-order reaction and other conditions which give measureable residual activity of enzyme without substrates, as well as a considerable change in activity (the signal of the experiment) after addition of substrates, is very important to get accurate results. Under our experimental conditions, which mimicked the buffer and protecting reagents in the catalytic reaction, ATP protected the enzyme effectively, while Mitra et al. (1970) found that no protection was afforded by ATP (2 mM) at pH 5.5, 50 °C, in their system. In fact, an alteration of binding behavior was also observed between pH 6 and pH 7.5 for ATP in our report.

In studying the enzyme–substrate interactions it is very important to use methods as direct as possible, e.g., a fluorescence study affords another and more direct means than steady-state kinetics.

This work was also aimed at the test of a more acceptable methodology for the study of enzyme–substrate interactions. After elucidation of the enzyme–substrate interactions, studies

on the ArgRS mechanism are being continued in this laboratory.

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Structure of a Hydroxyl Radical Induced Cross-Link of Thymine and Tyrosine†

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ABSTRACT: DNA–protein cross-links are formed when living cells or isolated chromatin is exposed to ionizing radiation. Little is known about the actual cross-linked products of DNA and proteins. In this work, a novel hydroxyl radical induced cross-link of thymine and tyrosine has been isolated along with a tyrosine dimer by high-performance liquid chromatography of aqueous mixtures of tyrosine and thymine that had been exposed to hydroxyl radicals generated by ionizing radiation. The isolated compounds have been examined by gas chromatography–mass spectrometry, high-resolution mass spectrometry, and ^1H and ^{13}C nuclear magnetic resonance spectroscopy. The structure of the thymine–tyrosine cross-link has been identified as the product from the formation of a covalent bond between the methyl group of the thymine and carbon 3 of the tyrosine ring. In addition, the 3,3′ tyrosine dimer was isolated and characterized. The mechanism of the formation of these compounds is discussed. This work presents the first complete chemical characterization of a hydroxyl radical induced DNA base–amino acid cross-link.

DNA–protein cross-links are formed when living cells or isolated chromatin in vitro is exposed to ionizing or UV radiation (Smith, 1976; Yamamoto, 1976; Mee & Adelstein, 1979). Although great effort has been spent on the study of this type of DNA damage, little is known about the actual cross-linked components of DNA and proteins in living cells [for a review, see Oleinick et al. (1986)]. In the case of DNA–protein cross-links in isolated chromatin, evidence indicates the involvement of hydroxyl radicals (OH radicals)[1] produced from water by ionizing radiation (Mee & Adelstein,

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[1] Abbreviations: HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; FT, Fourier transform; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TSP, sodium 4,4-dimethyl-4-silapentanoate-*2,2,3,3-d*$_4$; DOPA, 3-hydroxytyrosine; NOE, nuclear Overhauser effect.