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A fluorescence spectroscopic study of glutaminyl-tRNA synthetase from *Escherichia coli* and its implications for the enzyme mechanism

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Interaction between *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS) and its substrates have been studied by fluorescence quenching. In the absence of other substrates, glutamine, $tRNA^{Gln}$ and ATP bind with dissociation constants of 460, 0.22 and 180 μM, respectively. The presence of other substrates has either no effect or, at best a weak effect, on binding of ligands. Attempts to isolate enzyme-bound aminoacyl adenylate did not succeed. Binding of the phosphodiester, 5′-(methyl)adenosine monophosphate (MeAMP), to GlnRS was studied by fluorescence quenching and radioactive-ligand binding. tRNA also only has a weak effect on phosphodiester binding.

Selectively pyrene-labeled GlnRS was used to obtain shape and size information for free GlnRS. A comparison with the GlnRS shape in the GlnRS/$tRNA^{Gln}$ crystal structure indicates that no major change in shape and size occurs upon $tRNA^{Gln}$ binding to GlnRS. 5,5′-Bis(8-anilino-1-naphthalene sulfonate) (bis-ANS), a non-covalent fluorescent probe, was also used to probe for conformational changes in GlnRS. This probe also indicated that no major conformational change occurs upon $tRNA^{Gln}$ binding.

We conclude that lack of tRNA-independent pyrophosphate-exchange activity in this enzyme is not a result of either lack of glutamine or ATP binding in the absence of tRNA, or formation of aminoacyl adenylate and slow release of pyrophosphate. A conformational change is implied upon tRNA binding, which promotes pyrophosphate exchange. Fluorescence studies indicate that this conformational change must be limited and local in nature.

Aminoacyl-tRNA synthetases from *Escherichia coli*, as a group, catalyze reactions of identical chemical nature. The substrates of these enzymes, as a whole, are also very similar. Despite catalyzing chemical reactions that are similar, the primary and quarternary structures of the enzymes vary widely (Schimmel, 1987). There is, however, an underlying unity in the chemical mechanism of aminoacylation for most of the aminoacyl-tRNA synthetases, except arginyl-tRNA synthetase (ArgRS), glutamyl-tRNA synthetase (GluRS) and GlnRS (Ravel et al., 1965). The former group of enzymes catalyze tRNA-independent ATP/PP_i exchange in contrast to the latter group. The mechanistic and evolutionary implications of this deviation from the general pattern is not well understood.

ArgRS, GluRS and GlnRS are all small monomeric tRNA synthetases (Schimmel, 1987). The sequence of *E. coli* GluRS (Breton et al., 1986) and GlnRS (Yamao et al., 1982) are known. There is a considerable degree of sequence similarity, which is significantly more than the similarity found among other tRNA synthetases. In contrast to other synthetases, neither ArgRS nor GluRS catalyzed the formation of enzyme-bound aminoacyl adenylate (Kern and Lapointe, 1979; Crain and Peterkofsky, 1975). ArgRS and GluRS, however, differ in their substrate-binding properties. All three substrates are reported to bind more or less independently to the ArgRS (Lin et al., 1988), whereas in the case of GluRS, glutamic acid cannot bind in the absence of $tRNA^{Glu}$ (Kern and Lapointe, 1979). To our knowledge, no kinetic or substrate-binding study has been reported for the GlnRS from *E. coli*. Recently, an X-ray structure for GlnRS and its complex with $tRNA^{Gln}$ and ATP has been reported (Rould et al., 1989). This is the first reported structure of a tRNA/aminoacyl-tRNA synthetase complex. The wealth of structural information and availability of large amounts of the enzyme and tRNA, make it an ideal system for studying mechanistic aspects of aminoacylation and tRNA-dependent pyrophosphate exchange.

Aminoacylation of tRNA takes place on the 2′- or 3′-hydroxyl groups of the terminal adenosine (Freist, 1989). The last three nucleotides CpCpA are identical in all known tRNA. Although CpCpA has all the chemical requirements of a substrate for aminoacylation, e.g. it is not a substrate for phenylalanine-tRNA synthetase (Renaud et al., 1981), the question can be raised as to whether the rest of the tRNA molecule causes a conformational change in the enzyme, enhancing the aminoacylation activity. There has been reports of a tRNA-induced conformational change in the synthetases (Beresten et al., 1983; Bacha et al., 1982), although its relationship with the aminoacylation reaction is not clear (Ferguson and Yang, 1986). In the case of GluRS, ArgRS and GlnRS, the tRNA-induced conformational change assumes more importance, since tRNA binding triggers aminoacyl-adenylate formation and pyrophosphate exchange.

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Abbreviations. RS, tRNA synthetase; bis-ANS, 5,5′-bis(8-anilino-1-naphthalene sulfonate); BND–cellulose, benzoylated and naphthylated DEAE–cellulose; MeAMP, 5′-(methyl)adenosine monophosphate.

In this article, we report substrate binding, 5′-(methyl)adenosine monophosphate (MeAMP) binding and the influence of other substrates or products on the substrate-binding properties of this enzyme. We have also probed conformational differences of free and tRNA-bound GlnRS using fluorescence spectroscopy.

MATERIALS AND METHODS

MATERIALS

ATP, AMP, benzoylated and naphthylated DEAE-cellulose (BND-cellulose), ampicillin, cacodylic acid, streptomycin sulfate, and sucrose were from Sigma Chemical Company (St Louis, MO, USA). Sodium pyrophosphate and dicyclohexylcarbodiimide were from Aldrich Chemical Co. (Milwaukee, WI, USA). DEAE–Sephadex-A-25 was from Pharmacia United (Sweden). Phosphocellulose and DEAE–cellulose (DE-52) were from Whatman Inc. [^{3}H]Glutamine and [^{3}H]AMP were from Amersham (UK), and had specific radioactivities of 50 Ci/mmol and 13.8 Ci/mmol, respectively. Bactotryptone and yeast extract were from Difco Laboratories (Detroit, MI, USA). Pyrene maleimide and bis-ANS were purchased from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were of analytical grade.

METHODS

Enzyme assay

The protein concentration was measured using an $A_{280}^{1\%}$ of 10.2 (Hoben et al., 1982). GlnRS activity was measured by the rate of aminoacyl-tRNA formation. The aminoacylation was carried out in 62.5 mM potassium phosphate, pH 7.0, containing 12.5 mM $MgCl_2$, 2.5 mM ATP, 20 U/ml crude *E. coli* tRNA and 10 μM [^{3}H]glutamine. The enzyme was always diluted in 50 mM potassium phosphate, pH 7.0, containing 10% glycerol, 1 mg/ml bovine serum albumin and 10 mM 2-mercaptoethanol. After incubation at 37 °C for various times, 40 μl aliquots were withdrawn and applied to Whatman 3MM filter paper. After washing with cold trichloroacetic acid and ethanol, the precipitated radioactivity was determined by liquid scintillation counting.

Enzyme purification

Enzyme purification was basically as described by Soll and co-workers (Hoben et al., 1982) with minor modifications. A brief description is given below.

Growth of E. coli *harboring pMN20.* Cells harboring plasmid pMN20 were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin at 32 °C. Luria-Bertani medium contains 0.5% NaCl, 0.5% yeast extract and 1% tryptone. After growth to mid-log phase, the temperature was shifted to 42 °C and incubation continued for 4 h. The cells were then harvested by centrifugation.

Purification of GlnRS. All operations were performed at 4 °C. The cells were disrupted by sonication in 10 mM potassium phosphate, pH 8.0, containing 10% glycerol and 20 mM 2-mercaptoethanol. After streptomycin sulfate precipitation, the supernatant was chromatographed on a DEAE-cellulose column and eluted with a gradient of 100 ml 20 mM potassium phosphate, pH 7.2, and 100 ml 250 mM potassium phosphate, pH 6.5, in 20 mM 2-mercaptoethanol and 10% glycerol. It was then chromatographed on a phosphocellulose column and eluted with a 50 ml/50 ml gradient over 0–300 mM KCl in 10 mM potassium phosphate, pH 6.5, containing 20 mM 2-mercaptoethanol and 10% glycerol. The final specific activity was between 1.5–2.0 $\mu mol \cdot min^{-1} \cdot mg\ enzyme^{-1}$. This value is 15–20-times higher than that reported by Hoben et al. (1982), but comparable to that of Kern et al. (1980). The purified enzyme showed approximately 95% purity when analyzed by SDS/PAGE (Laemmli, 1970).

tRNA purification

Growth of E. coli *containing pRS3.* Cells harboring pRS3 were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin at 32 °C. After growth to mid-log phase, the temperature was shifted to 42 °C and incubation continued for 4 h (Perona et al., 1982).

Purification of tRNA. After harvesting, the cells were phenol extracted and ethanol precipitated. The precipitate was then fractionated on a DEAE-cellulose column to remove ribosomal RNA. The tRNA pool was then fractionated on a DEAE–Sephadex-A-25 column and eluted with a 500:500 ml gradient of 0.4 M NaCl, 8 mM $MgCl_2$ and 0.48 M NaCl, 16 mM $MgCl_2$ in 0.02 M Tris/HCl, pH 7.5, containing 1 mM sodium thiosulfate (Schejter et al., 1982). The appropriate fractions were ethanol precipitated, dialysed and further purified on a BND-cellulose column (Gillam et al., 1967), eluted with a 50 ml/50 ml gradient of 0.3–1.0 M NaCl in 0.1 M Tris/HCl, pH 7.5, containing 10 mM $MgCl_2$. The final specific activity was 1.2 nmol/A_{260}.

Synthesis of MeAMP

The synthesis was basically according ot Roy and Colman (1980), with methanol substituted for *n*-propanol. The product showed only one spot in thin-layer chromatography under ultraviolet light and the radioactivity comigrated with this spot. The product also showed an ultraviolet absorbance spectra identical to that of AMP, as expected.

Chemical modification

GlnRS was pyrene labeled by treatment with an eightfold molar excess of pyrenemaleimide. A *N,N*-dimethylformamide solution of pyrenemaleimide was added to 5 μM solution of GlnRS in 0.1 M Tris/HCl, pH 7.5, at 4 °C so that the final *N,N*-dimethylformamide concentration was less than 2% (by vol.). At different times, an aliquot was removed and quenched by adding 2-mercaptoethanol to a final concentration of 1 mM. It was then dialysed extensively against 0.1 M Tris/HCl, pH 7.5, and finally against the same buffer containing 6 M urea. The pyrene-labeled-GlnRS concentration was determined by the Bio-Rad protein assay (Bradford, 1976) using purified GlnRS in 6 M urea as a standard. The pyrene concentration was calculated using a molar absorption coefficient of 37500 M^{-1} cm^{-1} at 343 nm (Haugland, 1985). For the Perrin plot, a 1:1 molar ratio of pyrenemaleimide/GlnRS with a reaction time of 15 min was used and a Sephadex G-25 column was used for separation instead of dialysis.

Fluorescence methods

Steady-state fluorescence was measured in a Hitachi F-3000 spectrofluorimeter equipped with a computer for addition and subtraction of spectra. Bandwidths of both

emission and excitation monochromators were 5 nm unless stated otherwise. Background emission was corrected by subtracting signals from blank buffer.

Binding studies

The excitation and emission wavelengths were 295 nm and 340 nm, respectively, for binding studies of substrates, unless stated otherwise. In the cases of ATP and tRNA titrations, corrections for the inner-filter effect were made according to the formula: $F = F_{obs} \cdot \text{antilog}\,[(A_{ex} + A_{em})/2]$, where A_{ex} is the absorbance at the excitation wavelength, and A_{em} is the absorbance at the emission wavelength.

The absorbance at each point was measured in a Shimadzu UV-160 spectrophotometer in a cuvette of 1 cm pathlength at 25°C. Fluorescence-quenching data was either analyzed by a double-reciprocal plot of $1/\Delta F$ versus 1/[S] or by Eadie plot of ΔF versus ΔF/[S] (Lin et al., 1988).

Bis-ANS-binding studies were performed in 0.1 M Tris/HCl, pH 7.5, containing 15 mM $MgCl_2$. The excitation and emission wavelengths were 455 nm and 500 nm, respectively, unless mentioned otherwise. The excitation wavelength was chosen such as to avoid the inner-filter effect, even up to 100 μM bis-ANS. The binding isotherms were calculated by the method of Mas and Colman (1985).

Correlation times and limiting anisotropies were determined from isothermal Perrin plots (Cantor and Schimmel, 1980). Perrin plots was performed using 0.3 μM pyrene-labeled GlnRS in the absence or presence of equimolar concentration of $tRNA^{Gln}$. The viscosity of the solution was varied by inclusion of sucrose in the range 9–50% in 0.1 M Tris/HCl, pH 7.5, at 25°C.

Fluorescence lifetimes were determined in an Applied Photophysics single-photon-counting apparatus using a nanosecond lamp-based system. The lifetimes were obtained from decay curves by fitting to a single exponential function after deconvoluting the lamp-response function. The lifetimes of pyrene-labeled GlnRS were determined at 25°C in 0.1 M Tris/HCl, pH 7.5, containing 15 mM $MgCl_2$ at a protein concentration of 2.6 μM.

Attempted isolation of enzyme-bound aminoacyl adenylate

20 μl GlnRS (100 μg) was mixed with 80 μl 0.1 M Tris/HCl, pH 7.2, containing 50 mM $MgCl_2$, 10 mM ATP, 10 mM 2-mercaptoethanol, 200 μg/ml bovine serum albumin, 80 μM L-glutamine and 35 μCi L-[^{3}H]glutamine and incubated at 25°C for 5 min. The mixture was then loaded onto a Sephadex G-25 column (1.2 cm × 22 cm), previously equilibrated with 0.01 M sodium cacodylate, containing 0.5 mM EDTA and 0.05 M KCl, then eluted with the same buffer. The collected fractions were counted for radioactivity by liquid scintillation counting.

RESULTS

Fluorescence quenching or enhancement has been widely used to study ligand binding to proteins (Ward, 1985). In cases where the ligands do not fluoresce, quenching of protein internal tryptophan fluorescence upon ligand binding has been used to study protein-ligand interactions. Lin et al. (1988) have used both fluorescence and equilibrium dialysis to study ligand binding in *E. coli* ArgRS and obtained similar results. We have used quenching of protein fluorescence to study

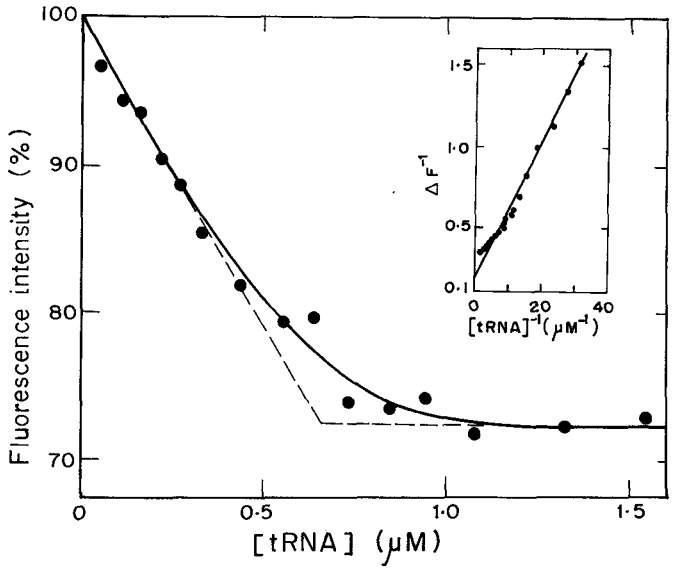

Fig. 1. *Determination of the dissociation constant and the stoichiometry of $tRNA^{Gln}$ binding to GlnRS.* Fluorescence titrations were in 0.5 μM GlnRS. The titration was performed in 0.1 M Tris/HCl, pH 7.5, containing 3 mM magnesium chloride. The abscissa value at the intersection between the tangent to the initial portion of the curve and the asymptotic line is equal to $n[E_t] + K_d$, where $[E_t]$ and n are the initial concentration and number of binding sites of the enzyme, respectively. The inset shows the double-reciprocal plot for titration of the GlnRS fluorescence with $tRNA^{Gln}$. The enzyme concentration was 0.02 μM. The titration was performed in 0.1 M Tris/HCl, pH 7.5, containing 3 mM magnesium chloride. F, fluorescence

tRNA, glutamine, ATP, pyrosphosphate and some combinations of the above ligands binding to GlnRS. We have also looked at the influence of ligand binding on the others tRNA synthetases.

Fig. 1 shows the quenching profile of protein fluorescence upon binding $tRNA^{Gln}$. Either K_d or the stoichiometry can be obtained from such plots, if the other is known. The dissociation constant was first determined at low GlnRS concentration (0.02 μM). The inset shows the double-reciprocal plot of the data. The dissociation constant of $tRNA^{Gln}$ in the presence of magnesium is 0.22 μM. This value is comparable to tRNA binding to ArgRS (Lin et al., 1988) and GluRS (Lapointe and Soll, 1972). A K_m has been reported for this enzyme by Ravel et al. (1965) but only in terms of the concentration of crude tRNA (mg/ml). From the data given in that article and the specific-activity data for the crude RNA used in that experiment, one can calculate a K_m of approximately 0.5 μM. This is close to the K_d reported here. The abscissa value of the intersection of the asymptotic line with the tangent to the initial portion of the quenching curve represents $n[E_t] + K_d$, where n is the number of $tRNA^{Gln}$-binding sites, E_t is the total enzyme concentration and K_d is the dissociation constant (Lin et al., 1988). From Fig. 1, using the value of K_d deduced previously, a value of 0.88 was obtained for n.

All the binding data are sumarized in Table 1. Binding of glutamine in the absence of magnesium is tighter (K_d = 0.15 mM) than in it's presence (K_d = 0.457 mM). The K_d of glutamine is comparable to the K_m value reported by Ravel et al. (1965) (K_m = 0.18 mM). The quenching of protein fluorescence by glutamine in the presence of saturating amount of tRNA and ATP shows a similar profile, although the extent of quenching varies (data not shown). Binding of tRNA causes

Table 1. *Substrate binding of GlnRS*
GlnRS concentrations were 1 μM unless mentioned otherwise. All binding studies were performed in 0.1 M Tris/HCl, pH 7.5, at ambient temperatures which was generally 25 °C. Magnesium concentrations were always 3 mM unless mentioned otherwise. In the glutamine-binding experiments, the $tRNA^{Gln}$ concentration was 1 μM and ATP concentration 5 mM. In the tRNA-binding experiments, protein concentrations were 1 μM when performed in the presence of ATP and glutamine. The binding constant reported for $tRNA^{Gln}$ in the presence of magnesium only, is an average of two values obtained at two different protein concentrations (0.15 μM and 0.02 μM). In the ATP-binding experiments, the glutamine concentration was 1 mM and the $tRNA^{Gln}$ concentration was 1 μM. The protein concentration was 0.5 μM when performed in the presence of magnesium and glutamine. In the pyrophosphate-binding experiments, the $tRNA^{Gln}$ concentration was 1.5 μM and AMP concentration was 2 mM

Substrates	Additions	Dissociation constant
		μM
Glutamine	none	150
	$MgCl_2$	460
	$MgCl_2/tRNA^{Gln}$	360
	$MgCl_2$/ATP	800
tRNA	$MgCl_2$	0.22
	$MgCl_2$/glutamine	0.52
	$MgCl_2$/ATP	0.7
ATP	$MgCl_2$	190
	$MgCl_2$/glutamine	160
	$MgCl_2/tRNA^{Gln}$	550
PP_i	$MgCl_2$	220
	$MgCl_2/tRNA^{Gln}$	510
	$MgCl_2$/AMP	390
	$MgCl_2$/AMP/$tRNA^{Gln}$	390
MeAMP	$MgCl_2$	71
	$MgCl_2/tRNA^{Gln}$	55

the glutamine K_d to be lowered slightly, whereas binding of ATP causes glutamine to bind approximately twofold weaker. The reported binding of arginine to ArgRS is considerably tighter than glutamine binding to GlnRS (Lin et al., 1988).

ATP binding to GlnRS is also characterized by fluorescence quenching (data not shown). The dissociation constant (0.186 mM) is somewhat lower than that for ArgRS (Lin et al., 1988). The dissociation constant is also of the same order of magnitude as the K_m reported by Ravel et al. (1965) (0.44 mM). K_m for ATP vary from 7 μM for ThrRS to 0.72 mM for LeuRS (Allende et al., 1970; Rouget and Chapville, 1968). The presence of glutamine has no effect on the binding of ATP to the enzyme.

Product dissociation is sometimes the rate-limiting step in enzyme catalysis (DiFranco, 1974). Tight binding and slow dissociation of pyrophosphate from the enzyme – aminoacyl-adenylate – pyrophosphate ternary complex could have a profound effect on the pyrophosphate-exchange rate. We have studied pyrophosphate binding in the presence of various ligands. The dissociation constant (0.22 mM) is marginally weakened in the presence of $tRNA^{Gln}$ (0.51 mM). ATP and glutamine also weakens the binding somewhat.

For aminoacyl-tRNA synthetases that catalyze tRNA-independent pyrophosphate exchange, it is possible to isolate enzyme-bound aminoacyl adenylate in the absence of tRNA. We have attempted to isolate GlnRS-bound glutaminyl adenylate under similar conditions. Only a small amount of

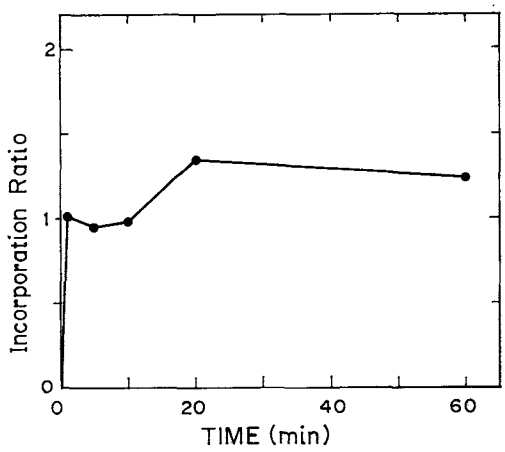

Fig. 2. *Incorporation of pyrenemaleimide into GlnRS at various times.* 5 μM GlnRS was mixed with 40 μM pyrenemaleimide in 0.1 M Tris/HCl, pH 7.5, at 4 °C. An aliquot was withdrawn at appropriate times and quenched with 1 mM 2-mercaptoethanol. The quenched reaction mixture was then dialysed as described in the Materials and Methods

radioactivity elutes in the void volume, where the enzyme-bound aminoacyl adenylate is expected (data not shown). An analysis based on the amount of enzyme applied to the column shows that radioactive isotope bound to enzyme in a molar ratio of 1:20.

Alkyl phosphodiesters of AMP bind to many aminoacyl-tRNA synthetases (Gross et al., 1979). They may be considered as analogs of aminoacyl adenylates. If the tRNA stabilises enzyme-bound aminoacyl adenylate, then it may have a similar effect on phosphodiesters. We have chosen MeAMP as the diester to avoid possible non-specific interaction of longer alkyl chains. Dissociation constants of 55 μM and 71 μM are obtained in the presence and the absence of $tRNA^{Gln}$, respectively. In order to confirm the above binding data, we have studied binding of Me-[^{3}H]AMP to GlnRS in the presence and absence of tRNA by the method of Hummel and Dryer (1962). A Sephadex G-25 column was equilibrated with 100 μM Me-[^{3}H]AMP and the enzyme applied to the column. Analysis of the excess radioactivity (i.e. the peak) in the void volume indicated less than 50% of that applied bound (data not shown). This agrees well with the binding data obtained by fluorescence quenching. In addition, MeAMP is found to be an inhibitor of the aminoacylation reaction (data not shown).

Sulfhydryl groups in proteins are favorite points of attachment of fluorescent probes and spin labels. A high degree of selectivity of sulfhydryl reagents and the general small number of sulfhydryl groups present in the proteins makes it possible to achieve a high degree of selectivity in labeling. To investigate the possibility of selective labeling of sulfhydryl groups, we have investigated the reaction of pyrenemaleimide with GlnRS.

Fig. 2 shows the incorporation of pyrenemaleimide as a function of reaction time. The incorporation ratio reaches approximately 1.2 groups modified/GlnRS molecule. Even at very early times, approximately one group is modified, followed by little further incorporation. This may mean that one group is reacting rapidly with pyrenemaleimide, followed by slow modification of one or more groups. Thus, pyrenemaleimide-modified enzyme with less than 1 mol pyrenemaleimide incorporated/mol enzyme may be considered as predominantly labeled at one group. Activity measurements

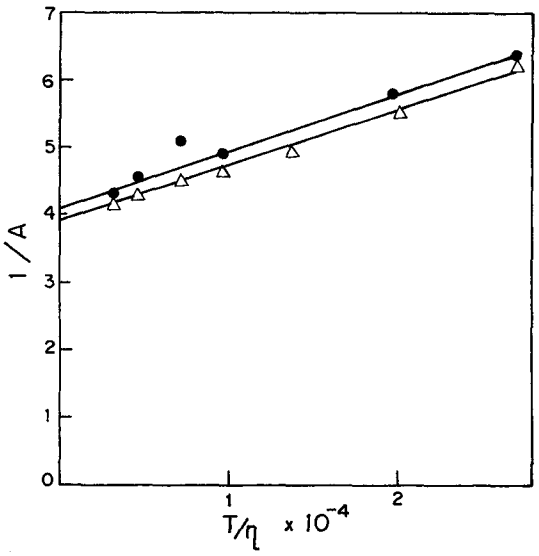

Fig. 3. *Perrin plot of pyrene-labeled GlnRS in the absence (●) and presence of (△) of tRNA^{Gln}.* Sucrose was included in the buffer to increase the viscosity. The pyrene-labeled GlnRS concentration was 0.3 μM and the tRNA^{Gln} concentration was 0.3 μM. The incubation was in 0.1 M Tris/HCl, pH 7.5, at 25 °C. A, anisotropy

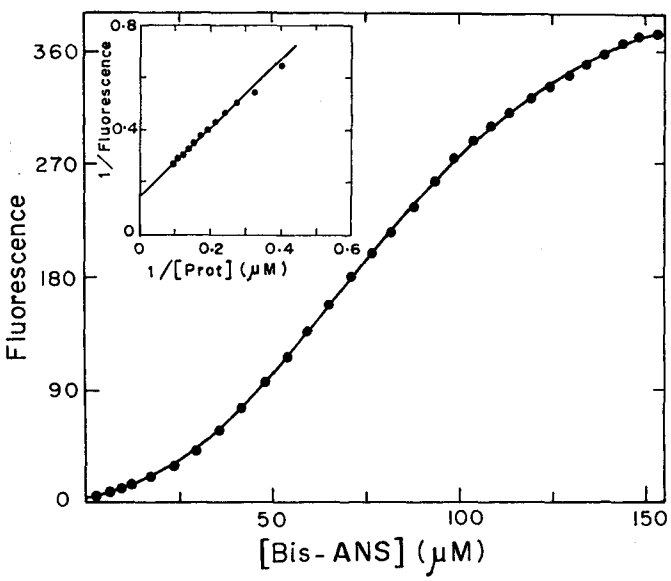

Fig. 4. *Titration of GlnRS with bis-ANS.* 5 μM GlnRS was titrated in 0.1 M Tris/HCl, pH 7.5, containing 15 mM $MgCl_2$. Excitation and emission wavelengths were 455 nm and 500 nm, respectively. The inset shows the reverse titration plot of 1 μM bis-ANS with increasing concentrations of GlnRS. The solution conditions were 0.1 M Tris/HCl, pH 7.5, containing 15 mM $MgCl_2$. Excitation and emission wavelengths were 455 nm and 500 nm, respectively. [Prot], total protein concentration

show that pyrenemaleimide-modified enzyme retains full catalytic activity (data not shown).

Utilizing the long lifetime of pyrene, we have determined the rotational correlation time of GlnRS and tRNA^{Gln}/GlnRS complex from the isothermal Perrin plot. Titration of GlnRS with tRNA^{Gln} results in a small fluorescence increase with a sharp end point at a GlnRS/tRNA^{Gln} ratio of 1:1. Analysis of the data indicated that tRNA^{Gln} binding is not significantly affected by pyrenemaleimide modification (data not shown). Fig. 3 shows the Perrin plot of both pyrenemaleimide-labeled GlnRS and pyrenemaleimide-labeled GlnRS/tRNA^{Gln} complex. The real-time fluorescence decay of the pyrene-labeled GlnRS can be fitted to a single exponential with a lifetime of 25.9 ns. Pyrenemaleimide-labeled proteins are known to exhibit biexponential behaviour with lifetimes ranging from 30 ns to greater than 100 ns (Howlaka and Hammes, 1977; Rao et al., 1979). In several cases, however, almost single exponential decays have been observed with lifetimes over 10–80 ns (Weltman et al., 1973). We could not detect any significant deviation from single exponential behaviour and the measured lifetime is close to that reported for some proteins. Using this lifetime and the slope of the Perrin plot, we have calculated the rotational correlation time of GlnRS to be 35 ns. A spherical protein of the same molecular mass should have a rotational correlation time of 27 ns (Cantor and Schimmel, 1980). The increased rotational correlation time is generally thought to occur because of the non-spherical nature of proteins. The ratio of the actual/expected correlation time can be related to the ratio of the two axes of the ellipsoid that describe the protein. For GlnRS, the actual/expected ratio for the correlation time is 1.3. This value yields an axial ratio of 3.75:1 (Benecky et al., 1990) and agrees well with that obtained for GlnRS in the GlnRS/tRNA^{Gln} complex. The hydrated molecular volume, calculated from the slope of the Perrin plot, is 170 nm^3. The calculated value of the long axis of the prolate ellipsoid would be 8.3 nm, assuming an axial ratio of 3.75:1. The value of the long axis again is in good agreement with the value determined from X-ray crystallography of the GlnRS/tRNA^{Gln} (Rould et al., 1989).

The rotational correlation time of the GlnRS/tRNA^{Gln} complex is 37.5 ns, which is very close to the expected value for a spherical protein of same molecular mass. This indicates that the complex is almost spherical in shape.

The above experiments with pyrenemaleimide-labeled GlnRS indicated that binding of tRNA^{Gln} does not lead to any major change in the shape and size of the molecule. Thus, any conformational change occurring upon tRNA binding must occur within the constraints of preserving the approximate overall shape and size of the molecule. Many conformational changes in proteins are characterized by domain movements without significantly changing the overall shape and size of the molecule. Bis-ANS is a non-covalent fluorescent probe which, in many cases, is known to bind to domain interfaces (Schneider et al., 1984) and is a sensitive monitor of environmental polarity and conformational changes in proteins (Rosen and Weber, 1969). We have studied bis-ANS binding to GlnRS and the influence of substrates on bis-ANS binding, to probe the nature of the conformational change, if any, upon tRNA^{Gln} binding. Fig. 4 shows the titration of GlnRS with increasing concentrations of bis-ANS. The initial fluorescence increase is modest, but at higher concentrations very high fluorescence values are reached. The inset shows the reverse titration, where a fixed concentration of bis-ANS is titrated with increasing concentrations of GlnRS. The double-reciprocal plot of the reverse-titration data is linear. A dissociation constant of 10 μM was obtained from the plot. The direct titration, however, showed a high degree of binding at considerably higher concentrations, indicating two types of binding sites. Fig. 5 shows the emission spectra of bis-ANS at two different drug/protein ratios. In one case, the drug concentration was kept low and a high protein concentration was used, so that bis-ANS binding to high-affinity sites are favored. In the other case, an excess-drug concentration over protein concentration was used, so as to cause significant

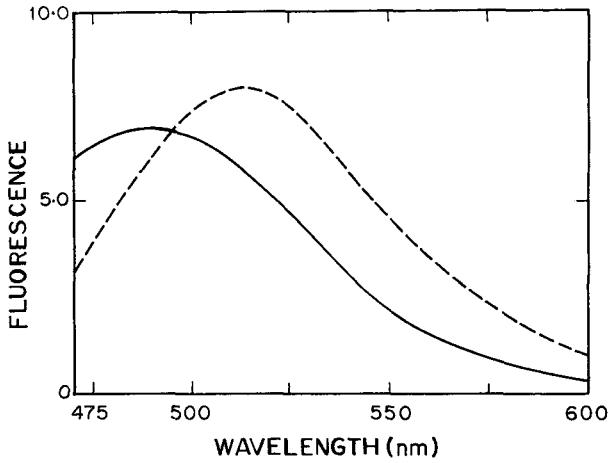

Fig. 5. *Fluorescence emission spectra of bis-ANS bound to GlnRS.* Spectra were recorded at an excitation wavelength of 385 nm with 1 μM bis-ANS and 10 μM GlnRS (——) and 455 nm with 50 μM bis-ANS and 1 μM GlnRS (— — —). Incubation was in 0.1 M Tris/HCl, pH 7.5 containing 15 mM $MgCl_2$ at 25°C

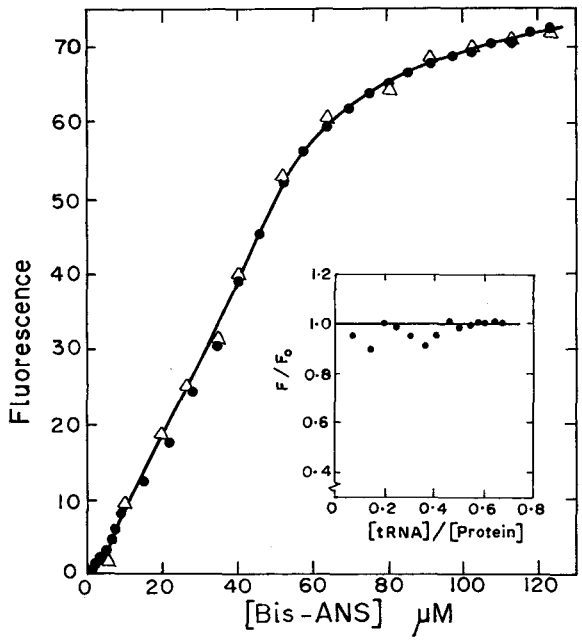

Fig. 6. *Titration of GlnRS with bis-ANS in the presence (△) and in the absence (●) of $tRNA^{Gln}$.* 1 μM GlnRS was used in both experiments and 1 μM $tRNA^{Gln}$ was used in the experiment performed in the presence of $tRNA^{Gln}$. The incubation was in 0.1 M Tris/HCl, pH 7.5, containing 15 mM $MgCl_2$ at 25°C. The inset shows the influence of tRNA on the high-affinity bis-ANS-binding site of GlnRS. 10 μM GlnRS and 1 μM bis-ANS in 0.1 M Tris/HCl, pH 7.5, containing 15 mM $MgCl_2$, was titrated with increasing concentrations of $tRNA^{Gln}$. The excitation and emission wavelengths were 385 nm and 500 nm, respectively. F in the inset defines fluorescence value in the presence of tRNA and F_0 refers to the fluorescence value in the absence of tRNA

binding to lower-affinity sites. If there is only one class of binding site, identical spectra would be obtained in both cases (Lawson and York, 1987). The emission spectra under the two different conditions have substantially different emission maxima, indicating two different types of binding sites are present. Binding analysis of the high-affinity binding site at high protein concentration indicated a binding constant of 11.5 μM and stoichiometry of 0.9.

In order to determine the effect of $tRNA^{Gln}$ binding to two bis-ANS sites, we have followed the bis-ANS binding in the presence of $tRNA^{Gln}$, under conditions where binding occurs predominantly at either high-affinity or low-affinity sites. Fig. 6 shows the bis-ANS titration of GlnRS, in the presence and absence of $tRNA^{Gln}$, at a high drug/protein ratio. The titration curve is virtually identical in the presence and absence of $tRNA^{Gln}$. The inset shows the effect of tRNA binding on the fluorescence of bis-ANS, when conducted at a high protein/drug molar ratio. Under this condition, where bis-ANS is expected to bind at the high-affinity site, the addition of $tRNA^{Gln}$ to an equal concentration of GlnRS has very little effect on the bis-ANS fluorescence, indicating that $tRNA^{Gln}$ has no effect on either K_d or the quantum yield of the bound bis-ANS. Thus, as in the case of the low-affinity bis-ANS-binding site, very little effect on either K_d or the quantum yield could be seen at the high-affinity site.

DISCUSSION

E. coli GlnRS is representative of the group of synthetases that do not catalyze tRNA-independent pyrophosphate exchange. There are several possibilities that could account for the inability of the enzyme to catalyze tRNA-independent pyrophosphate exchange. (a) Either ATP or glutamine does not bind in the absence of tRNA. This is probably the case of GluRS (Kern and Lapointe, 1979). (b) Aminoacyl adenylate forms, but the enzyme-bound pyrosphosphate exchanges very slowly with the external pyrosphosphate. (c) Aminoacyl-adenylate formation does not take place until the tRNA binds. (d) Aminoacyl adenylate is not an intermediate in the aminoacylation of $tRNA^{Gln}$.

We have looked into the binding of glutamine, ATP and $tRNA^{Gln}$ to GlnRS by quenching the enzyme's internal fluorescence. It is clear that both the ATP and glutamine bind to the enzyme in the absence of tRNA with dissociation constants of 0.19 mM and 0.46 mM, respectively. The dissociation constants are close to the reported K_m for this enzyme. tRNA has very little influence on the binding of ATP and glutamine, indicating that lack of ATP or glutamine binding in the absence of tRNA is not the cause of lack of pyrophosphate exchange. In this respect, GlnRS is similar to ArgRS and different from GluRS. This is also consistent with the work by Ravel et al. (1965), in which GluRS showed tRNA-independent pyrophosphate exchange at high glutamate concentrations, whereas no such activity was detectable for GlnRS, even at very high glutamine concentrations.

We have attempted to isolate enzyme-bound aminoacyl adenylate by gel filtration, under conditions where enzyme-bound aminoacyl adenlyate could be isolated for other aminoacyl-tRNA synthetases (Emmett and Schimmel, 1972). No significant amount of enzyme-bound radioactivity could be detected. This rules out the possibility that aminoacyl adenylate forms, but enzyme-bound pyrophosphate exchanges very slowly to prevent any pyrophosphate exchange. In addition, as reproted above, pyrophosphate binds only weakly to the enzyme. Even if the association rate constant is well below the diffusion-controlled limit (e.g. $10^4\ M^{-1}\ s^{-1}$), the dissociation rate constant of $2.20\ s^{-1}$ would still be high enough for a significant amount of pyrophosphate exchange to occur. This makes it unlikely that slow exchange of bound

pyrophosphate with the external pyrophosphate could be the cause of the inability of the enzyme to catalyze tRNA-independent pyrophosphate exchange.

The third and the fourth possibilities are more difficult to exclude or prove directly. We have looked into the influence of tRNA binding on the binding of phosphodiester MeAMP. Like other substrates, tRNA has very little influence on binding of MeAMP. If MeAMP is a good analog of aminoacyl adenylate, then binding of tRNA does not influence the stability of bound aminoacyl adenylate. Thus, a tRNA-assisted formation of aminoacyl adenylate and subsequent formation of aminoacyl-tRNA or concerted reaction in the quarternary complex (enzyme-tRNA-ATP-glutamine) is the likely explanation of the lack of tRNA-independent pyrophosphate exchange with this enzyme.

tRNA-induced formation of aminoacyl adenylate may imply a tRNA-induced conformational change. We have looked into the possibility of a tRNA-induced conformational change in GlnRS at two levels. GlnRS was first labeled with pyrene, then the rotational correlation time was determined by an isothermal Perrin plot. The axial ratio and length of the long axis calculated for a prolate ellipsoid matches with that obtained by X-ray crystallography of the $tRNA^{Gln}$/GlnRS complex. This indicates that no major change in shape takes place upon $tRNA^{Gln}$ binding.

Bis-ANS is a non-covalent fluorescent probe which binds to hydrophobic sites in proteins (Wu and Wu, 1978). In some cases, it binds to domain interfaces and acts as a sensitive monitor of conformational changes. Bis-ANS binds to GlnRS at a high-affinity site with a stoichiometry of 1, and to lower-affinity binding sites with higher stoichiometry. None of the sites are affected significantly by $tRNA^{Gln}$ binding. Although one cannot rule out a conformational change on the basis of lack of an effect on two different binding sites of a fluorescent probe, the data taken together with previous experiments indicates that no major change in the enzyme structure occurs upon tRNA binding. Thus, aminoacyl adenylate formation and pyrophosphate exchange triggered by $tRNA^{Gln}$ binding may result from limited conformational changes that are transparent to the methods used here.

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