Biophysical Chemistry, 48 (1993) 159–169
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 01798

Characterization of aminoacyl-adenylates in *B. subtilis* tryptophanyl-tRNA synthetase, by the fluorescence of tryptophan analogs 5-hydroxytryptophan and 7-azatryptophan

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(Received 15 May 1993; accepted in revised form 14 July 1993)

Abstract

The tryptophan analogs 5-hydroxytryptophan (5HW) and 7-azatryptophan (7AW) are capable of being biosynthetically incorporated into bacterial proteins and can be used as intrinsic fluorescence probes of protein structure, function and dynamics. A prerequisite for analog incorporation is their recognition by tryptophanyl-tRNA synthetase (TrpRS) and the formation of the analog aminoacyladenylate in the enzyme's active site. The binding of 5HW and 7AW to *B. subtilis* TrpRS and the stability of the corresponding aminoacyladenylates of 5HW and 7AW were examined using their unique spectroscopic properties. The adenylate of 7AW in the active site of TrpRS exhibited intense fluorescence with a 10.5 ns fluorescence decay time. Enzyme-bound 7AW-adenylate was a long-lived intermediate with a half-life of over 9 hours. Enzyme-bound 5HW-adenylate fluorescence was quenched compared to that of 5HW in solution. The 5HW-adenylate/TrpRS complex was much less stable than that of 7AW, with a half-life of 33 minutes. Rapid hydrolysis of the 5HW-adenylate may explain the apparent proofreading observed which prohibits 5HW incorporation into proteins in the presence of tryptophan. Hydrolysis of the adenylates of both analogs restored the fluorescence parameters towards those of the analogs in solution. Neither 1-methyltryptophan nor 5-methoxytryptophan were capable of forming long-lived aminoacyladenylate intermediates in TrpRS. This study provides perspectives on the usefulness of 5HW and 7AW as intrinsic fluorescence probes of protein structure. The enhanced fluorescence of 7AW suggests its location in a buried hydrophobic environment in the protein. Exposure to water results in significant fluorescence quenching. These studies clearly demonstrate the utility of Trp analogs for the elucidation of molecular details of protein structure and dynamics.

Keywords: Fluorescence decay time; Protein-protein interactions; Protein folding; Alloprotein; Aminoacyl-tRNA synthetase

1. Introduction

The tryptophan (Trp) analogs 5-hydroxytryptophan (5HW) and 7-azatryptophan (7AW) were identified as being capable of incorporation into proteins in early experiments [1–3]. Biosynthetic incorporation of Trp analogs into proteins indicates that the analog amino acid or its derivatives is a substrate for each of the enzymatic steps involved in protein synthesis. The critical first step is the recognition of the L-amino acid and

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formation of the activated aminoacyladenylate. The enzyme tryptophanyl-tRNA synthetase (TrpRS) first catalyzes the reversible acylation reaction:

$$\text{TrpRS} + \text{Trp} + \text{ATP} \rightleftharpoons \text{TrpRS(Trp-AMP)} + \text{PP}_i \quad \text{(I)}$$

The tryptophanyladenylate (Trp-AMP) is stable in the active site of the enzyme, but is very unstable in solution at pH > 5 [4]. Reaction I is driven to form the Trp-AMP/enzyme complex by hydrolysis of pyrophosphate (PP_i) by inorganic pyrophosphatase. Finally, $tRNA^{Trp}$ reacts with the enzyme bound Trp-AMP, forming tryptophanyl-$tRNA^{Trp}$ (Trp-$tRNA^{Trp}$).

$$\text{TrpRS(Trp-AMP)} + \text{tRNA}^{\text{Trp}} \rightleftharpoons \text{TrpRS} + \text{Trp-tRNA}^{\text{Trp}} + \text{AMP} \quad \text{(II)}$$

It has been shown that 7AW can be incorporated into *E. coli* proteins [1] in studies of bacterial growth in media containing 7AW. The analog 7AW was toxic to bacteria and unable to sustain cell growth. This toxicity of Trp analogs is often found and has been attributed to incorporation of the analog into essential proteins which become inactivated [1–3], as a result of local structural defects. However, several bactericidal toxic analogs of Trp are capable of undergoing incorporation into bacterial proteins. These include fluorinated tryptophans [5–7], azatryptophans [1,8,9], 5-methyltryptophan [3] and 5HW [3,10]. Bovine pancreatic TrpRS has been used to study the ATP activation of as several Trp analogs *in vitro* [2,11,12]. With "bait-and-switch" techniques [9,10,13] and the use of Trp auxotrophs of *E. coli* it is possible to produce useful quantities of proteins having toxic Trp analogs incorporated in place of native Trp. Such modified proteins have been shown to be useful in UV absorbance, fluorescence and ^{19}F NMR spectroscopic studies of protein structure.

The spectroscopic advantage of both 5HW and 7AW is that these amino acids have red shifted absorbancies which allow their selective fluorescence excitation in the presence of other proteins containing normal Trp [10,13–15]. Their incorporation into proteins implies that they must form an aminoacyladenylate in bacterial TrpRS. Hence an analog that forms a stable, enzyme-bound aminoacyladenylate may possibly aminoacylate tRNA for subsequent biosynthetic incorporation [16] into proteins.

This work examines the feasibility of using the unique spectroscopic properties of the selected Trp analogs to detect and characterize their aminoacyladenylates in *B. subtilis* TrpRS. The demonstration of the existence of aminoacyladenylate/enzyme complexes can be used to determine the feasibility of whether a specific analog can eventually be incorporated into an expressed protein without having to conduct a biosynthetic incorporation experiment. Since the enzyme-bound aminoacyladenylates can undergo rapid irreversible hydrolysis [4] after dissociation from the enzyme, the dissociation and hydrolysis kinetics of the aminoacyladenylate can be followed by changes in the fluorescence parameters of the Trp analog.

2. Methods and materials

Purification of *B. Subtilis* TrpRS from an *E. coli* expression system essentially followed the methods described by Shi et al. [17]. An additional gel filtration step used a 1.1 m × 2.5 cm column of Sephacryl S-200 HR (Pharmacia) equilibrated at pH 7.0 (10 m*M* Pipes, 10 m*M* $MgCl_2$, 10 m*M* NaCl, and 100 m*M* KCl). Solutions of TrpRS for fluorimetry were prepared from lyophilized enzyme dissolved at high concentrations (typically 5 mg/ml) in a pH 8.6 reaction buffer (200 m*M* Tris-HCl, 20 m*M* MgAcetate). Substrates used for reaction with enzyme; Na_2 ATP, NaAMP, Na_2pyrophosphate, D,L-7-azatryptophan, L-5-hydroxytryptophan, D,L-5-methoxytryptophan (all from Sigma), and D,L-1-methyltryptophan (Aldrich) were used without further purification. [γ-^{32}P]ATP and [α-^{32}P]ATP were obtained from American Radiolabelled Chemicals Inc. Reactions to form the enzyme-bound aminoacyladenylate [18] were carried out at 23°C for 5 min in the presence of a dialysis button (6000 mol. wt. cutoff) containing 5 units of inorganic pyrophosphatase (Sigma). 500 μl of reac-

tion mixture contained 100 μM TrpRS, 200 μM L- or 400 μM D,L-Trp analog, and 400 μM ATP. After a 5 min reaction time, 250 μl of the reaction mixtures was passed through a 15 cm column of Sephadex G-25 fine (Pharmacia) equilibrated with freshly prepared pH 7.5 10 mM K_2HPO_4, 100 mM KCl buffer, which was also the buffer used for all spectroscopic studies reported.

Solutions for fluorimetry were diluted (if required) to a final absorbance of 0.10 or less at the excitation wavelength to avoid any inner filter effect. 2-Aminopyridine (Eastman Kodak) was used without purification as a quantum-yield standard (0.66, [19]) at 310 nm excitation in 0.1 N H_2SO_4 purged with nitrogen for 10 min prior to measurement. Absorbance spectra were collected using a Varian DMS 200 spectrophotometer with a 1 nm bandpass. Steady-state fluorescence spectra were collected with Glan Taylor polarizers oriented to eliminate anisotropic effects, using an SLM 8000C instrument with 4 nm emission and excitation bandpass. Spectra were corrected for the blank contribution and for wavelength dependence of the instrument response.

Fluorescence decay data was measured using the method of time-correlated single photon counting (TCSPC) using laser/microchannel plate instrumentation. This instrument and data analysis techniques have been reported in detail earlier [20,21]. All measurements were performed at 20°C with 4 nm bandpass, and corrections were made from the blank containing buffer only. The instrument response function was determined from the Raman scattering of the excitation by water at 346 nm for 310 nm excitation. Data were collected at 10 or 20 ps/channel in 2048 channels. Each decay curve contained $(1–2) \times 10^6$ counts, except where indicated. The time-resolved data was combined with the corrected steady-state spectra to derive the Decay Associated Spectra (DAS) [22].

3. Results and discussion

3.1. Aminoacyladenylates in TrpRS

In a double-labelling experiment with *E. coli* TrpRS, Merle et al. [18] showed that short incubation of reagents followed by Sephadex G-25 chromatography would produce stoichiometric amounts of TrpRS(Trp-AMP). Similar conditions were used in this report for the preparation of the adenylates of the Trp analogs with the *B. subtilis* enzyme. A typical elution profile of a reaction mixture of TrpRS, L-tryptophan or analog, and ATP has three well resolved peaks corresponding to the enzyme, unreacted ATP, and unreacted Trp, respectively. A by-product of the desired *in vitro* reaction involving a second molecule of ATP (Reaction III) has been described [23,24], where the ribose hydroxyl of ATP is acylated in a manner analogous to the acylation of the 3′-terminal adenosine of tRNA. This slow reaction [18] requires an

$$\text{TrpRS(Trp-AMP)} + \text{ATP} \rightarrow \text{TrpRS(Trp-ATP)} + \text{AMP} \qquad \text{(III)}$$

excess of ATP. It was necessary to demonstrate the absence of the enzyme bound acyl-transfer Trp-ATP by-product, hence preparations of TrpRS(Trp-AMP) were made using [γ^{-32}P]ATP and [α^{-32}P]ATP. The reaction mixtures contained 26 μM TrpRS, 200 μM L-Trp and 400 μM labelled ATP. No radioactivity (counts = background) eluted with the TrpRS peak when [γ^{-32}P]ATP was used. With [α^{-32}P]ATP, a 0.5 ml fraction of the first peak contained 2.1 ($\pm$0.3 μM) [α^{-32}P]ATP and 2.3 μM TrpRS. The absence of radioactivity from the [γ^{-32}P] of ATP indicated that no ATP was associated with the enzyme, and therefore no enzyme bound Trp-ATP acyl-transfer by-product was formed under the conditions used to produce TrpRS(Trp-AMP).

The absorption spectra of Trp analogs 7AW and 5HW (Fig. 1A) and analogs 1-methyltryptophan (1MW) and 5-methoxytryptophan (5MeOW) (Fig. 1B) show an extended low energy absorbance compared to Trp itself. It has already been shown that 7AW and 5HW are useful as new intrinsic fluorescence probes in proteins because they are both capable of exclusive excitation in the presence of Trp [10,14]. Since 1MW and 5MeOW have significant absorbance > 300 nm they too may be candidates for use as intrinsic fluorescent Trp analogs. Experiments were

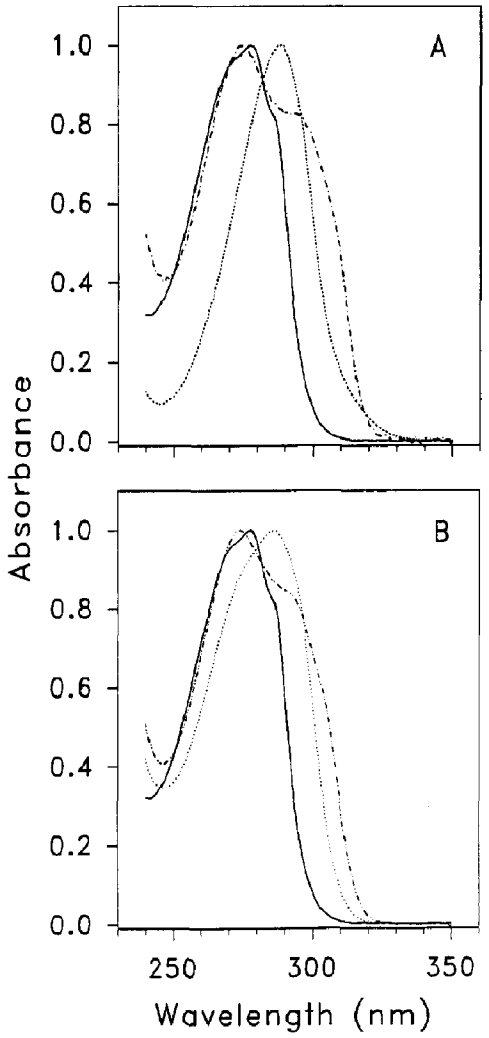

Fig. 1. Peak normalized absorbance spectra of Trp analogs used in this study. (A) L-Trp (———), L-5-hydroxytryptophan (·–·–·); and D,L-7-azatryptophan (·····). (B) L-Trp (———); D,L-5-methoxytryptophan (– · –); and D,L-1-methyltryptophan (·····).

conducted with all these analogs to determine whether they associated in stoichiometric amounts with TrpRS to form the Trp aminoacyladenylate.

Each of the four Trp analogs was used in the aminoacyladenylate reaction with TrpRS and ATP and the reaction mixture was chromatographed on G-25. The absorption spectra shown in Fig. 2 are those of the enzyme eluant of the reaction with 5HW and 7AW. Compared to the enzyme alone there was an increase in the absorbance at 310 nm due to the 5HW or 7AW and

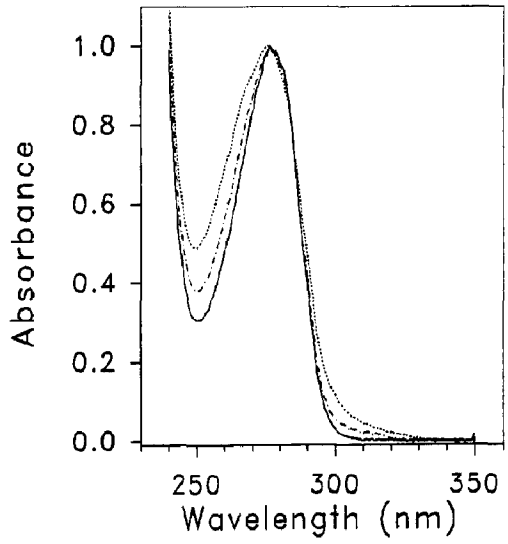

Fig. 2. Peak normalized absorption spectra of TrpRS after reaction with various analogs and chromatographic separation as shown in Fig. 1. TrpRS alone (———); 16 μM TrpRS with 10.5 μM 7-azatryptophanyladenylate bound (·····); 17 μM TrpRS with 3.6 μM 5-hydroxytryptophanyladenylate bound (·–·–·). Concentrations were obtained from linear combinations of individual absorbancies of TrpRS, 5HW, 7AW and AMP at 310 and 280 nm, together with the absorbancies of the complexes after pyrophosphate addition.

an increase at 260 nm due to the absorbance of the adenine in the aminoacyladenylate/TrpRS complex. Since 7AW has a lower extinction coefficient than 5HW at 310 nm (Table 1) it would be expected that the 310 nm shoulder of the TrpRS(5HW-AMP) would be higher than that of TrpRS(7AW-AMP) if both adenylates were present in equal stoichiometric amounts. However,

Table 1

Extinction coefficients for stock solutions of substrates in pH 7.5 10 mM K_2HPO_4, 100 mM KCl

Analog	ϵ_{310} (M^{-1} cm^{-1})	ϵ_{280} (M^{-1} cm^{-1})
L-5HW	2450	4900
DL-7AW	1205	5080
DL-5MeOW	1590	5140
DL-1MeW	340	3847
AMP	0	2310
TrpRS	100	24154 [a]

[a] Protein concentration determined by amino acid analysis, within 2.3% of the value calculated as per Gill et al. [38].

the absorbance at 310 nm and at 260 nm was greater for the TrpRS(7AW-AMP) complex indicating that more of it formed than TrpRS(5HW-AMP).

The ratio of free enzyme to that of the TrpRS–aminoacyladenylate complex could be determined in the following way. The eluant mixture of enzyme and TrpRS–aminoacyladenylate complex was treated with PP_i which hydrolysed the complex to the original unreacted constituents of enzyme, ATP, and Trp analog. The concentration of ATP and the Trp analog were considered to be equal. The absorption spectrum was then measured and from the extinction coefficients at 280 nm and 310 nm of each constituent (Table 1) the ratio could be determined. For TrpRS(7AW-AMP) the ratio of unreacted enzyme to complex was 1.5:1, while for TrpRS (5HW-AMP) the ratio was 4.7:1. The lower amount of apparent formation of the TrpRS (5HW-AMP) complex may be due to either its slower rate of formation according to reaction I and/or a faster rate of dissociation and subsequent hydrolysis of the released 5HW-AMP.

The absorption spectra and fluorescence spectra of the enzyme eluant of 1MW indicated that 1MW did not form a stable aminoacyl-adenlyate complex with the enzyme. In the case of 5MeOW there was some evidence from the absorption and fluorescence spectra that there was some association of the 5MeOW with the enzyme. However, when PP_i was added to the eluent in order to reverse the reaction there was no change in the fluorescence behaviour as was observed for the reactions with 7AW and 5HW.

Whether 5MeOW-AMP or even 1MW-AMP is even transiently formed in the ezymatic reaction may best be detected by either successful $tRNA^{Trp}$ aminoacylation by 5MeOW, or by the biosynthetic incorporation of 5MeOW into bacterial proteins. This possibility is currently under investigation. The apparent inability to form 1MeW-AMP suggests that the indole N–H of substrate Trp may be required for enzymatic recognition by

Table 2

Steady-state and time-resolved fluorescence decay parameters

Sample	ϕ [a]	λ_{max} (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_2	SVR
TrpRS(7AW-AMP) [b]	0.348	361	10.5 ±0.004	1.06 ±0.01		0.925	0.075		1.69
TrpRS(7AW-AMP) [c]			10.6 ±0.004	0.915±0.01		0.855	0.145		1.91
TrpRS(7AW-AMP)+PP_i	0.017	391 [d]	10.0 ±0.019	0.825±0.002	0.260±0.007	0.048	0.803	0.149	1.78
7AW	0.013	402		0.757±0.001	0.242±0.003 [e]		0.977	0.023	1.33
TrpRS(5HW-AMP) [f]	0.148	334	3.80±0.004	1.15 ±0.008		0.623	0.377		1.84
TrpRS(5Hw-AMP)+PP_i [g]	0.179	336	3.80±0.004	1.25 ±0.02		0.830	0.170		1.91
TrpRS(5HW-AMP) [h]			3.76±0.003	1.13 ±0.007					1.92
5HW	0.254	336	3.60±0.001			1.0			1.87
TrpRS [i]			4.46±0.02	1.20 ±0.02	0.072±0.001	0.045	0.038	0.917	1.68

[a] Φ is the quantum yield, τ_1 is the fluorescence decay time with preexponential term α_i. SVR is the serial variance ratio reflecting goodness of fit of the global analysis, "ideal" fits correspond to SVR of 2. 310 nm excitation, preexponentials at 400 nm for 7AW; 330 nm for 5HW samples.

[b] Global analysis of complete spectrum 325–450 nm.

[c] Global analysis of spectrum 360–450 nm avoiding enzyme fluorescence.

[d] Broad maximum centred at 375 nm (Fig. 3C).

[e] Pre-exponential became negative at wavelengths > 400 nm.

[f] Sample undergoing hydrolysis during data aquisition (Fig. 5B).

[g] Decay parameters of sample hydrolyzed after 3 h without PP_i were the same. (Fig. 5C).

[h] Global analysis of fluorescence decay at 330 nm vs. time depicted in Fig. 6.

[i] See emission spectra (Fig. 3B). Less than 350000 counts/decay curve.

TrpRS, probably through hydrogen-bond interactions.

3.2. 7-Azatryptophanyladenylate

The fluorescence spectrum of the TrpRS (7AW-AMP) complex was dramatically different from that of an aqueous solution of 7AW itself. There was a 27 fold increase in the ϕ_f changing from 0.013 in aqueous solution to 0.348 in the complex (Table 2). A 30 nm blue shift of the spectral maximum (Fig. 3A) was also observed. After an excess of PP_i was added to the TrpRS(7AW-AMP) complex the fluorescence spectrum (Fig. 3A) resembled that of a mixture of the enzyme and the free amino acid, 7AW. When the chromophore 7AW is exposed to aqueous solvent it has a very low ϕ_f. As a result the fluorescence due to a 4:1 mixture of TrpRS and DL-7AW shows that the fluorescence of TrpRS Trp-92 can be observed below 400 nm, even when excited at 310 nm. Figure 3B shows the fluorescence of this mixture together with the individual fluorescence spectra of the enzyme and DL-7AW with 310 nm excitation. The absorbance of 20 μM TrpRS at 310 nm excitation was 0.002, but the quantum yield of the single Trp-92 residue was 0.14. The absorbance of 80 μM DL-7AW was 0.100, and its quantum yield was 0.013. A measure of the sensitivity of a fluorophore can be approximated by the product of the extinction coefficient and the quantum yield. At 310 nm, this value is 16 M^{-1} cm^{-1} for 7AW, and 14 M^{-1} cm^{-1} for TrpRS. Owing to the high sensitivity of TCSPC fluorescence decay measurements it is possible to resolve decay times that are due to Trp fluorescence at $\lambda < 380$ nm, even at 310 nm excitation.

The steady-state fluorescence of the TrpRS (7AW-AMP) decreased with time from that found immediately after elution of the complex from the G-25 column. A semi-logarithmic plot of this loss of fluorescence intensity at 20°C is presented in Fig. 4 (the sample was left in the dark between fluorescence readings). A straight line fit of this plot indicated first order kinetics. The fluorescence decrease is attributed to the slow rate of dissociation of the 7AW-AMP from the active site, followed by the rapid hydrolysis of the adenylate. The half-life of the TrpRS(7AW-AMP) complex calculated from this plot [25] is 9.2 hours. 7AW-AMP is thus very tightly held by TrpRS in the absence of PP_i. This shows that the high-en-

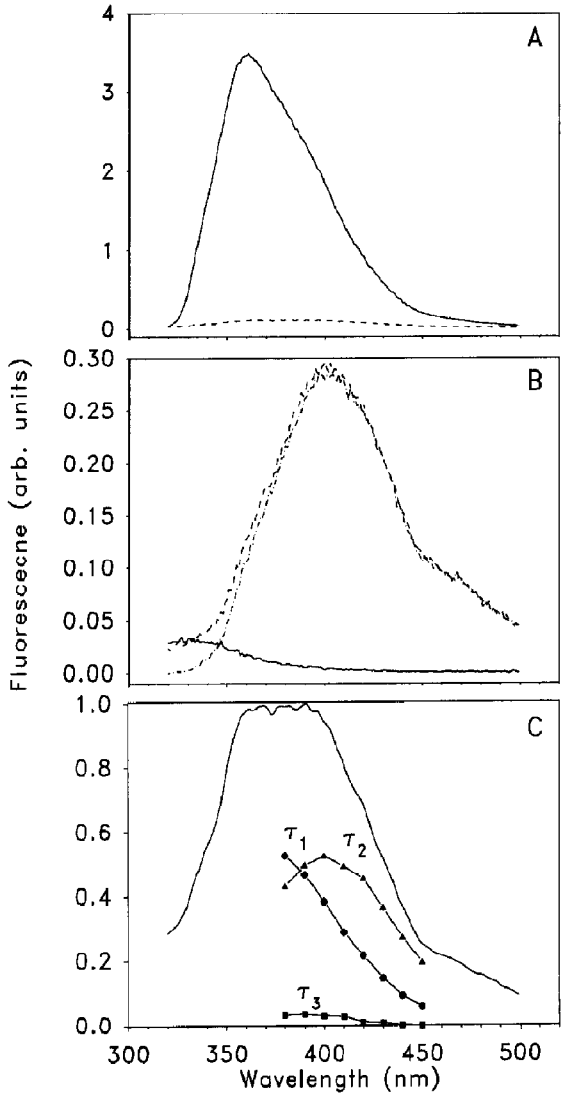

Fig. 3. Corrected steady-state fluorescence emission spectra at 310 nm excitation. (A) TrpRS-bound 7-azatryptophanyladenylate (———); and the same material with a two-fold excess of inorganic pyrophosphate (------). (B) 20 μM TrpRS (———); 80 μM D,L-7-azatryptophan (·–·–·); Mixture of 20 μM TrpRS and 80 μM D,L-7-azatryptophan (------). (C) Decay associated spectra (DAS) of the spectra shown in A. TrpRS-bound 7-azatryptophanyladenylate with a two-fold excess of inorganic pyrophosphate.

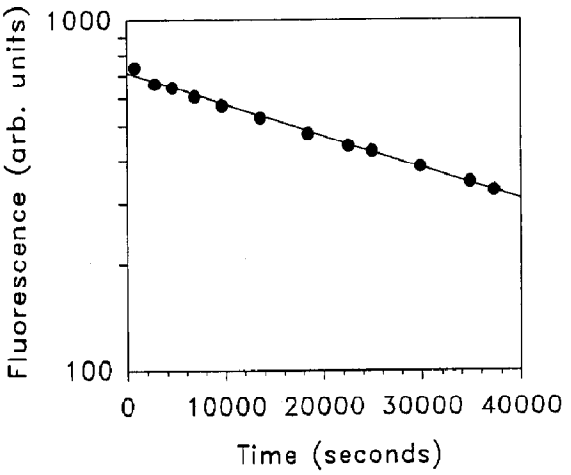

Fig. 4. Semilogarithmic plot of the decline in fluorescence intensity of enzyme bound 7-azatryptophanyladenylate with time. The half-life of TrpRS-bound 7-azatryptophanyl-adenylate is 552 min.

ergy activated amino acid is held for a extremely long time in the active site, conserving the energy of amino acid activation until $tRNA^{Trp}$ forms a complex with the enzyme and is aminoacylated.

The time-resolved fluorescence of the TrpRS (7AW-AMP) complex (Table 2) measured shortly after G-25 elution could best be fit to a biexponential decay process across the spectrum. At all wavelengths the majority of the fluorescence (> 98%) could be attributed to a long decay time, 10.5 ns component. The decay time of the second decay component, which accounted for the balance of the fluorescence intensity, was different at $\lambda < 360$ nm than from that found at $\lambda \geq 360$ nm. A global analysis [22] which included all the datasets gave only a modest fit (SVR = 1.69) to two exponentials. When this analysis was extended to a fit to three-exponential decay components, the decay times were 10.79 ± 0.02 ns, 5.9 ± 0.14 ns and 0.83 ± 0.01 ns (SVR = 1.95). The 5.9 ns component did not make a significant contribution to the fluorescence above 360 nm. Another global analysis which included only datasets measured at $\lambda \geq 360$ nm gave a fully satisfactory fit (SVR = 1.91) to two exponential decay components, with the shorter decay time being 0.915 ns. The 5.9 ns component is attributed the long decay time component of the Trp-92 fluorescence from the enzyme, which is poorly defined owing to its small contribution to the total fluorescence. The 0.83 ns component is assigned to another of the Trp-92 fluorescence decay components as well as to the small amount of free 7AW which resulted from adenylate hydrolysis. The long lifetime component is clearly that of 7AW in the TrpRS(7AW-AMP) complex.

When an excess of PP_i was added to the solution of the complex, three decay components were well defined (Table 2). In these experiments only data from $\lambda > 380$–450 nm were measured, since it was known that the Trp-92 fluorescence made an important contribution to the fluorescence signal at $\lambda < 380$ nm. Global analysis (Table 2) of the data allowed the presentation of the DAS of the PP_i treated material is presented in Fig. 3C. The two sub-nanosecond decay times can be assigned to free 7AW, being very similar to decay times reported for 7AI in water [26]. A long fluorescence decay time of 10 ns (0.56% of the fluorescence of the intact complex) was still observed contrary to expectations as it was thought that it would disappear after PP_i was added. While the majority of the fluorescence resulting from what was assigned to the TrpRS (7AW-AMP) complex disappeared this long 10 ns lifetime component was not removed by the addition of an even larger excess of PP_i. Time-resolved fluorescence measurements of incubations of TrpRS with stoichiometric amounts of combinations of AMP, 7AW, Mg^{2+} and PP_i were performed to see if this component could be attributed to the equilibrium binding of 7AW into the active site. A long decay time component could not be observed. Several additional experiments were performed in order to try to account for this small amount of 10 ns fluorescence component. It was not due to any acyl-transfer products involving free AMP. It is suggested that it results from a very small amount of aggregated protein which has "trapped" 7AW-AMP. Another important conclusion of these combination experiments is that there was no spectroscopic evidence of 7AW binding in the absence of ATP. This was consistent with the sequential mechanism of ATP binding before Trp, as shown by the inhibition studies of *B. subtilis* TrpRS kinetics of Xu et al. [27].

A recent series of experiments with 7-azaindole (7AI) [14,15,26,28–31] has illustrated the complex photochemistry of 7AW. 7-Azaindole is capable of undergoing a tautomerization reaction catalyzed by solvent hydroxyl groups as found in water and alcohols [15,26,28]. In aprotic solvents the quantum yield of 7-azaindole is large, e.g. 0.38 in acetonitrile [26]. In water and alcohols, the 7AI quantum yield is very small, e.g. 0.032 in H_2O. In the experiments reported herein the significantly high level of 7AW fluorescence in the TrpRS(7AW-AMP) complex is suggested to be due to the binding of 7AW-AMP into the hydrophobic [27] L-Trp specific binding pocket of the enzyme. The decay time, emission maximum and quantum yield of buried 7AW-AMP most closely match those of 7AI and 1Me7AI in aprotic solvents such as acetonitrile and diethyl ether [26], rather than those of 7AI in alcohols. The results indicate that hydrogen bonding of the indole N–H of substrate Trp-AMP may be important in the binding of the adenylate in the enzyme.

There are two other examples of the use of 7AW fluorescence to study proteins [8,15]. In an early report Schlesinger showed that 7AW incorporated in alkaline phosphatase [8] had an intense, blue-shifted fluorescence in buffer. Upon denaturation in 6 *M* guanidine-HCl, the fluorescence intensity decreased, becoming more like the free amino acid. More recently, Negrerie et al. [15] reported experiments of a peptide containing 7AW which interacted with a protease resulting in a change in the fluorescence decay time from 870 ps to 675 ps and a blue shift of the fluorescence maximum when bound to protein. These results were explained as being consistent with the similar changes observed for 7AI in a less polar alcohol. The decrease in decay time was attributed to the binding of the 7AI side chain into a hydrophobic pocket of the enzyme and the inability of forming hydrogen bonding complexes involving N1 and N7 with H_2O.

The observations reported herein, indicate that when 7AW is located in a hydrophobic pocket or core of protein, the formation of N1 and N7 hydrogen bonded complexes with water is prevented. This results in a significant increase of fluorescence intensity with a concomitant increase in the excited state decay time, and blue shift of the emission maximum. This implies that the interpretation of Negrerie et al. [15] requires modification. Our observations suggest that either H_2O is not entirely excluded from the binding pocket of *Streptomyces griseus* proteinase B, or this pocket contains polar residues which may hydrogen bond to the 7AW residue causing tautomerization. It is known that this protease apparently indiscriminately binds several bulky hydrophobic amino acid side chains such as Trp, Tyr or Leu [15].

It is further suggested that the high fluorescence of 7AW-substituted alkaline phosphatase [8] in Schlesinger's work must be due to protection of one or more of the seven 7AW residues in the protein from solvent in the folded protein. Unfolding with guanidine-HCl results in the decrease of this fluorescence as the 7AW residues become exposed to H_2O where they may undergo tautomerization. This rationalization of Schlesinger's results suggests that fluorescence changes of proteins with 7AW incorporated can provide a useful and specific biophysical probe for the examination of the kinetics of protein folding.

3.3. 5-Hydroxytryptophanyladenylate

The fluorescence spectra ($\lambda_{ex} = 310$ nm) of the TrpRS(5HW-AMP) complex measured immediately after elution from G-25 chromatography, and that after the addition of excess PP_i is shown in Fig. 5A. The fluorescence of the freshly eluted TrpRS(5HW-AMP) complex was slightly shifted (2 nm) and quenched when compared to that of free 5HW (Table 2). The fluorescence of TrpRS(5HW-AMP) complex changed rapidly with time and by analogy with the TrpRS(7AW-AMP) complex this was attributed to the dissociation and hydrolysis of the 5HW-AMP. The time resolved fluorescence of the TrpRS(5HW-AMP) complex decayed with double exponential decay kinetics showing decay times of 3.80 ns and 1.15 ns (Table 2). This compares with single exponential decay kinetics for 5HW in aqueous solution ($\tau = 3.60$ ns). The double exponential decay behaviour is interpreted as indicating that the

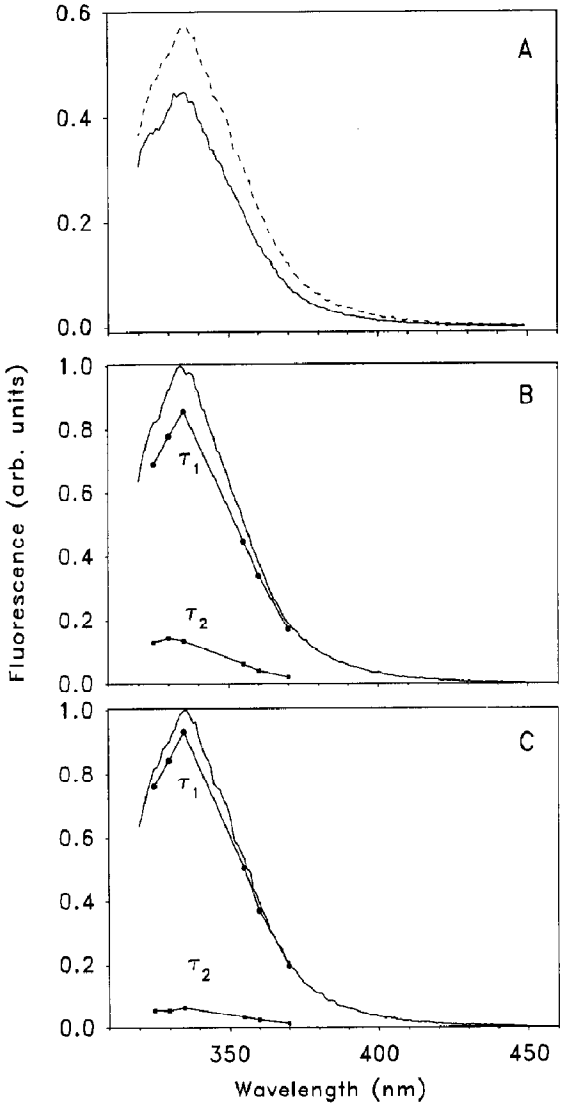

Fig. 5. (A) Corrected steady-state fluorescence emission spectra at 310 nm excitation. TrpRS-bound 5-hydroxytryptophanyladenylate (———); and the same material with a 2-fold excess of inorganic pyrophosphate (------). (B, C) Decay associated spectra of (B) The decaying TrpRS-bound 5-hydroxytryptophanyladenylate. (C) TrpRS-bound 5-hydroxytryptophanyladenylate with a two-fold excess of inorganic pyrophosphate.

5HW-AMP is in two conformational states within the enzyme complex. From the DAS of the TrpRS(5HW-AMP) complex (Fig. 5B) it can be seen that the 3.80 ns component represents 62% of the 5HW-AMP and provides 85% of the total fluorescence. The spectral maximum of the 1.15 ns component was found at 330 nm, which is significantly shifted from that of the 3.80 ns component (340 nm). After the addition of excess PP_i, the shorter lifetime was not entirely removed (Table 2, Fig. 5C) but may be due to a small constant amount of enzyme fluorescence which may still result from excitation at 310 nm. The 3.80 ns decay time in this case may represent a weighted average of the 3.60 ns decay time of free 5HW and the 4.46 ns decay time of the enzyme. This indicates that a small component of the fluorescence of the TrpRS(5HW-AMP) complex may be due to that of the enzyme itself and hence the proportions of the decay components would require adjustment.

Labouesse and co-workers have used the fluorescence quenching of Trp upon the formation of Trp-AMP to follow the kinetics and stoichiometry of *E. coli* [32,33] and bovine pancreas TrpRS [34]. By measuring the time-resolved fluorescence at 330 nm at time intervals after elution from the G-25 chromatogram the progress of the release of 5HW-AMP from the TrpRS(5HW-AMP) complex could be monitored. The temporal change in fractional fluorescence of each of the two decay components together with the increase in the steady state fluorescence are shown in Fig. 6. The dotted line in Fig. 6 represents the fractional fluorescence of the short decay time that could not be removed by excess PP_i and was attributed to TrpRS Trp-92 fluorescence. Analysis of a semi-logarithmic plot of this data where the final fractional fluorescence attributed to the enzyme was subtracted from the fractional fluorescence of the short decay time for each time point gave a half-life of 33 min for the TrpRS(5HW-AMP) complex. This was very much shorter than that of TrpRS(7AW-AMP) complex.

Aminoacyl-tRNA synthetases can undergo editing or proofreading of noncognate amino acids [35,36]. Pretransfer proofreading occurs when the noncognate aminoacyladenylate is hydrolyzed prior to tRNA aminoacylation. This is monitored indirectly by determining the ratio of AMP generated to tRNA aminoacylated [27]. Excess AMP generated is evidence of hydrolytic proofreading. The spectral properties of 5HW and 7AW have allowed us to directly monitor the loss of their TrpRS aminoacyl-adenlyate com-

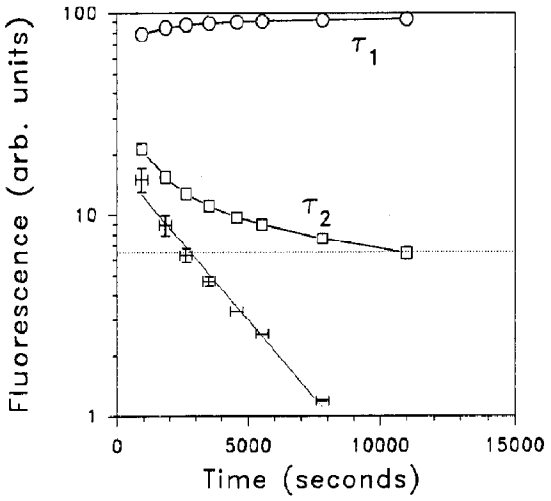

Fig. 6. Semi-logarithmic plot of the change in 5-hydroxytryptophanyladenylate fluorescence with time. Percent fractional contribution of the τ_1 component (○) and the τ_2 component (□) with time. Dotted line represents residual protein fluorescence contribution to τ_2. The dissociation of the TrpRS(5HW-AMP) complex is assigned to the fractional fluorescence of τ_2 above that of the dotted baseline. Horizontal error bars represent the fluorescence decay collection time. Vertical error bars represent the change in steady-state fluorescence during the measurement period.

plexes. The TrpRS(5HW-AMP) complex is 16 times more likely to dissociate and hydrolyse than the TrpRS(7AW-AMP) complex.

5-Hydroxytryptophan is a natural amino acid but, to the best of our knowledge, has not been found in the amino acid primary sequence of any natural protein. In higher eucaryotes 5HW is a precursor to serotonin (5-hydroxytryptamine). Early experiments with bovine pancreatic TrpRS showed that 5HW was not a substrate [2]. A mammalian TrpRS could be expected to more strongly discriminate against 5HW due to the larger amounts of 5HW present from the production of serotonin. It is possible that the bacterial tryptophanyl-tRNA synthetase has also experienced some evolutionary pressure against its reaction with 5HW. *In vivo* proofreading of *B. subtilis* TrpRS against 5HW was demonstrated using a Trp auxotroph of *B. subtilis* [3]. In that study, 5HW incorporation halted with the addition of $\frac{1}{10}$ the concentration of Trp. A pretransfer hydrolytic proofreading mechanism of TrpRS may serve to prevent mischarging of $tRNA^{Trp}$ with 5HW in the presence of Trp. The inefficient formation of the TrpRS(5HW-AMP) complex and rapid loss of any 5HW-AMP adenylate which did form supports this understanding.

3.4. 5HW and 7A W as intrinsic fluorescence probes of proteins

Both these Trp analogs have unique spectral properties which can enhance the information available from protein fluorescence experiments. The ability to selectively excite either analog in the presence of other proteins containing Tyr or Trp residues has significant potential for an improved understanding of the molecular details of protein–protein and protein–nucleic acid interactions. It has also been demonstrated that 5HW is a superior probe of fluorescence anisotropy than Trp itself since anisotropy values close to the theoretical limit of 0.4 can be observed [10]. This has been attributed to the separation of the ^1L_a and ^1L_b transition dipoles of the 5-hydroxyindole ring [37]. The ^1L_b dipole is responsible for the extended red absorption spectra of 5HW. Recently it has been shown that the ^1L_a and ^1L_b dipoles of 7AI overlap across its excitation spectrum [31]. In addition the low fluorescence yield and subnanosecond decay time of exposed 7AW reduces its utility as a fluorescence anisotropy probe when located in such a protein domain.

On the other hand these experiments and those reported much earlier [8] indicate that 7AW undergoes very large changes in its fluorescence properties on going from a polar to a non polar environment. 7AW has the potential for large changes in fluorescence. The longer decay time of a buried 7AW residue renders it more useful for dynamic studies with fluorescence anisotropy. This polarity dependence of the 7AW fluorescence could be used to monitor protein refolding events as well as conformational changes and ligand binding.

In this work we have shown that it is possible to obtain a preliminary determination of the potential for incorporation of Trp analogs in proteins. The experiments with the Trp analogs as TrpRS substrates have shown the potential of their use as mechanistic probes of TrpRS func-

tionality. The growing use of such analogs demonstrates their significant potential in protein studies.

Acknowledgements

The technical expertise of Mr. D.T. Krajcarski is appreciated in this work. We gratefully acknowledge the assistance of Dr. J.T. Wong and in making the expression system for TrpRS available to us.

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