2-p-TOLUIDINYLNAPHTHALENE-6-SULFONATE, A FLUORESCENT REPORTER GROUP FOR L-ISOLEUCYL-tRNA SYNTHETASE[1]

Eggehard Holler, Edward L. Bennett and Melvin Calvin

Laboratory of Chemical Biodynamics
Lawrence Radiation Laboratory
University of California
Berkeley, Calif. 94720

Received August 10, 1971

SUMMARY

The binding of 2-p-toluidinylnaphthalene-6-sulfonate to L-isoleucyl-tRNA synthetase is associated with a strong increase of fluorescence intensity and a concomitant shift in the wavelength of maximum emission. Binding of the fluorophore does not interfere with substrate binding nor the ATP-$^{32}PP_i$ exchange activity of the enzyme. The formation of binary and ternary enzyme-substrate complexes is indicated by a significant quenching of the fluorescence Dissociation constants were determined from the degree of quenching as a function of the substrate concentration. Evaluation of spectrofluorometric data suggest that substrate binding is associated with a conformation change in which tryptophan side chains of the protein are involved.

The intrinsic fluorescence of L-isoleucyl-tRNA synthetase has been reported to be inert to the binding of the substrates L-isoleucine, ATP^{4-} and $P_2O_7^{4-}$ (1). With the intention to investigate the catalytic action of L-isoleucyl-tRNA synthetase by fast reaction techniques, we have found a suitable fluorescent probe in 2-p-toluidinylnaphthalene-6-sulfonate.* In this communication we report results from equilibrium measurements investigating the binding of TNS and the various substrates.

---

[1]This research was supported, in part, by the U.S. Atomic Energy Commission. E.H. is at present a Helen Hay Whitney Fellow.

*Abbreviation: TNS = 2-p-toluidinylnaphthalene-6-sulfonate.

Table I

Dissociation constant and fluorescence properties of the L-isoleucyl-tRNA synthetase-TNS complex at pH 8.2. The dissociation constants were evaluated as indicated in Fig. 2. The enhancement factors f are calculated for the increase of the fluorescence intensity when a TNS molecule is transferred from water into the enzyme-TNS complex. When excited at 290 nm instead of 366 nm, the enhancement factor increases, presumably because of energy transfer from the protein to TNS.

Temperature (°C)	$\lambda_{ex.}$ (nm)	$\lambda_{em.}$ (nm)	K_D (10^{-5} M)	f
10	290	470		4500
	366	450	4	1800
25	290	470		1300
	366	470	7	500

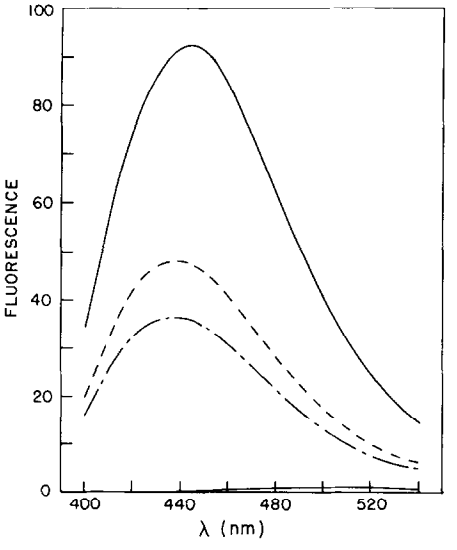

Fig. 1

Emission spectra for TNS in water (bottom solid line), for L-isoleucyl-tRNA synthetase-TNS complex (upper solid line), for L-isoleucyl-tRNA synthetase-TNS-L-isoleucine complex (– – –) and for L-isoleucyl-tRNA synthetase-TNS-L-isoleucine-$P_2O_7^{4-}$ complex (— - — -) at pH 8.2, 10°C. Measurements were performed using a Hitachi-Perkin-Elmer fluorometer MPF-2A. Excitation wavelength was 340 nm with 10 nm slit width in the excitation and emission light path. The spectra are not corrected for instrument properties. Initial concentrations were 1.1 μM TNS, 2.3 μM L-isoleucyl-tRNA synthetase, 0.3 mM L-isoleucine, 0.7 mM $P_2O_7^{4-}$. L-isoleucyl-tRNA synthetase had been highly purified from *E. coli B* according to the method previously described (8). TNS was obtained from Sigma as Lot 60C-5270. All experiments reported in this communication were done in the presence of 0.01 M 2-mercaptoethanol and potassium phosphate, ionic strength 0.05.

When dissolved in water, TNS fluoresces weakly at a wavelength of maximum emission close to 500 nm (2). In the presence of L-isoleucyl-tRNA synthetase, fluorescence increases strongly (Table I) with a concomitant shift of the maximum emission down to 443 nm (Fig. 1). The excitation spectrum is similar to the spectrum reported previously (2) except that an additional peak at 287 nm (uncorrected) is observed which is presumably associated with the absorbance for tryptophan side chains.

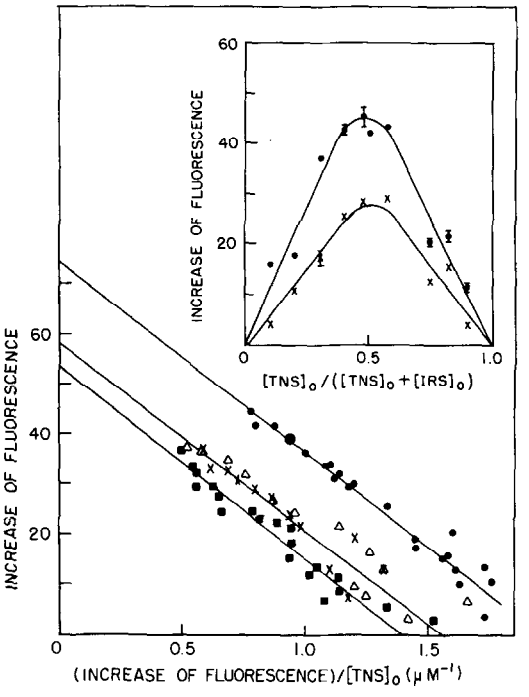

Fig. 2

Benesi-Hildebrand Plot (4) for the determination of the dissociation constant of the complex between TNS and L-isoleucyl-tRNA synthetase (•), TNS and L-isoleucyl-tRNA synthetase-L-isoleucine complex (x), TNS and L-isoleucyl-tRNA synthetase-ATP^{4-} complex (□), TNS and L-isoleucyl-tRNA synthetase-$P_2O_7^{4-}$ complex (Δ). Evaluation is based on the equation $\Delta F = \Delta F_\infty - K_D \cdot \Delta F/[TNS]$. The symbols ΔF, ΔF_∞ and K_D refer to the increase in fluorescence at the concentration [TNS], to the fluorescence increase when all enzyme is complexed with TNS, and to the dissociation constant of the enzyme-TNS complex, respectively. Fluorescence had been corrected for light absorbance using the method applied by McClure and Edelman (9). Wavelengths were 366 nm for excitation and 450 nm for emission. Solutions were of pH 8.2 at 10°C. Initial concentrations were 2 μM to 72 μM TNS, 0.4 μM L-isoleucyl-tRNA synthetase, 0.5 mM L-isoleucine, 3 mM ATP^{4-}, and 2.5 mM $P_2O_7^{4-}$. The stoichiometry of the binding of TNS is determined from the plot in the inset, according to the method of Job (3). Wavelengths were 366 nm for excitation and 470 nm for emission. Concentrations of TNS and the enzyme were varied over a 10-fold range while the sum of the concentrations had the fixed value 3.3 μM. Crosses refer to the presence of 0.31 mM L-isoleucine.

TNS binds in a 1:1 stoichiometry to the enzyme as indicated by the position of the maximum at 0.5, according to the method of Job (3) in Fig. 1, inset. The dissociation constant and fluorescence properties of the enzyme-TNS complex were evaluated from Fig. 2. In the inset, the data were plotted according to the method of Benesi and Hildebrand (4), and the dissociation constant was calculated from the slope of the line which gave the best fit to the experimental data. The fluorescence intensity of the enzyme-TNS complex is taken from the intercept and is compared with the fluorescence of free TNS (Table I).

The following experimental data indicate that TNS does not bind to the active site: 1. The binding of substrates does not affect the stability of the enzyme-TNS complex. This can be seen from Fig. 2; the lines in the presence of the various substrates L-isoleucine, ATP^{4-}, and $P_2O_7^{4-}$ are parallel to each other, indicating the same dissociation constants for the enzyme-TNS complexes. 2. Saturating amounts of TNS do not affect the ATP-$^{32}PP_i$ exchange activity of the enzyme (Fig. 3). However, a binding locus which overlaps the site involved in the tRNA charging reaction cannot be excluded.

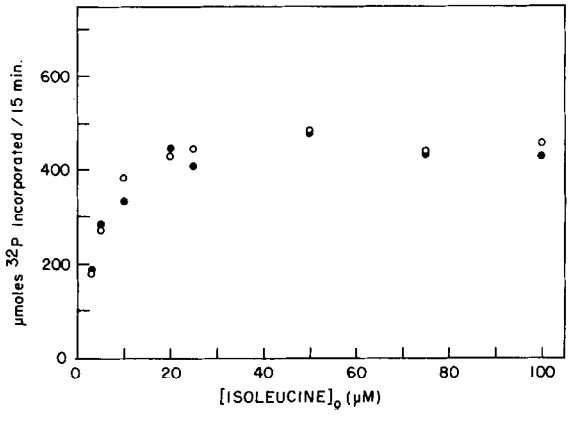

Fig. 3

Examination of the effect of TNS on the ATP-$^{32}PP_i$ exchange activity of L-isoleucyl-tRNA synthetase. Filled circles refer to the presence of 0.11 mM TNS. The exchange was measured at pH 8.2 and 25°C following the method described by Calendar and Berg (10). Solutions contained 8 x 10^{-9} M enzyme and the same buffer solution used for the fluorescence measurements.

Binding of the substrates L-isoleucine, ATP^{4-}, $P_2O_7^{4-}$, $MgATP^{2-}$ and $MgP_2O_7^{2-}$ is associated with partial quenching of the fluorescence (Fig. 4). Concomitantly, the wavelength of maximum emission is shifted to a still lower position at 437 nm (Fig. 1). Control experiments using glycine and L-alanine confirmed that quenching is not an unspecific effect (Fig. 4).

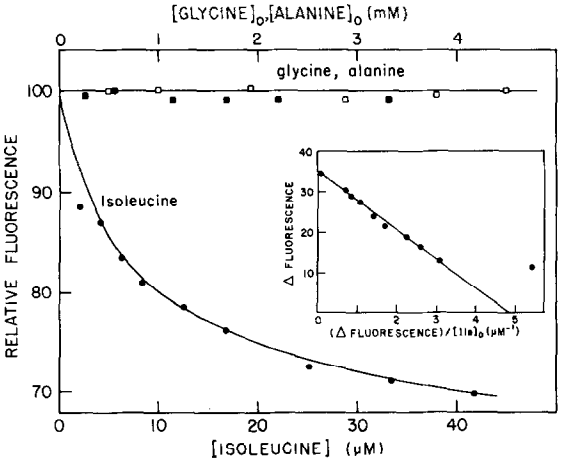

Fig. 4

Quenching of the fluorescence of L-isoleucyl-tRNA synthetase-TNS complex upon binding of L-isoleucine (●) as a function of concentration. Glycine (□) and L-alanine (■) do not interact with the enzyme. The data in the inset were plotted according to the method of Benesi and Hildebrand (4) for evaluation of the dissociation constant and of the degree of quenching when all enzyme is saturated with substrate. Solutions at pH 8.2 and 10°C were of 0.4 μM enzyme, 5 μM TNS and 3.8 mM $MgCl_2$. Wavelengths were 366 nm for excitation and 470 nm for emission.

The dissociation constants for the various substrates have been evaluated from the degree of quenching as a function of the substrate concentration using the method of Hildebrand and Benesi (4), Fig. 4, inset. The enzyme-TNS-substrate complexes were also characterized by the degree of maximum quenching in relation to the fluorescence intensity of the enzyme-TNS complex. Data are listed in Table II.

The technique is suitable for the investigation of the formation of ternary enzyme-substrate complexes under the condition of saturation by the first substrate. The fluorescence quenching for the second substrate is smaller

Table II

Binding of L-isoleucine, ATP^{4-}, $P_2O_7^{4-}$, $MgATP^{2-}$ and $MgP_2O_7^{2-}$ to L-isoleucyl-tRNA synthetase. Dissociation constants K_S and maximum fluorescence quenching relative to fluorescence intensity of the enzyme-TNS complex, $\Delta F/F$, were evaluated from Benesi-Hildebrand plots (4). Binding was investigated as a function of the order in which two substrates were added to the reaction mixture. The first substrate was present at saturating concentrations. The data represent average values for at least two determinations, and experimental errors refer to mean deviations. Maximum quenching was determined for 366 nm excitation and 470 nm emission wavelengths. Values in parentheses refer to excitation at 290 nm. Initial concentrations were at pH 8.2, 1-10 μM TNS and 0.05-1.0 μM enzyme.

Temperature (°C)	Substrate	Order	K_S (M)	$\Delta F/F$ (%)
10	L-isoleucine	1	$(7 \pm 2) \times 10^{-6}$	42 ± 7 (60 ± 3)
25		1	$(5.8 \pm 0.8) \times 10^{-6}$	37 ± 6 (68 ± 1)
10	ATP^{4-}	2	$(1.0 \pm 0.3) \times 10^{-4}$	35 ± 5
10	L-isoleucine	1		
10	$P_2O_7^{4-}$	2	$(2.5 \pm 1.0) \times 10^{-4}$	25 ± 5
10	ATP^{4-}	1	$(1.4 \pm 0.4) \times 10^{-4}$	45 ± 10
25		1	$(2.5 \pm 0.3) \times 10^{-4}$	40 ± 8 (67 ± 6)
25	L-isoleucine	2	2×10^{-6}	10
10	ATP^{4-}	1		
10	$P_2O_7^{4-}$	2	---	0
25	$MgATP^{2-}$	1	$(1.75 \pm 0.5) \times 10^{-4}$	30 ± 5
10	$P_2O_7^{4-}$	1	$(0.3 \pm 0.2) \times 10^{-4}$	40 ± 3
25	$P_2O_7^{4-}$	1	$(2.6 \pm 0.7) \times 10^{-4}$	40 ± 5 (70 ± 5)
10	L-isoleucine	2	$(10 \pm 3) \times 10^{-6}$	30 ± 6
25	MgP_2O_7	1	$(1.9 \pm 0.5) \times 10^{-4}$	30 ± 4
10	Mg^{2+}	1	---	0
10	L-isoleucine	2	$(7.7 \pm 0.8) \times 10^{-6}$	37 ± 2

than for the first substrate and becomes smaller at higher temperatures. We have investigated the binding as a function of the sequence by which the substrates are added to the reaction mixture. It can be seen from Table II that the sequence of addition has no effect. $P_2O_7^{4-}$ added to ATP^{4-} does not produce quenching, and vice versa, indicating competitive binding. ATP^{4-} and $P_2O_7^{4-}$ on the one hand and L-isoleucine on the other hand bind independently. Temperature has only a moderate effect on the stability of the complexes. The data are similar to those which have been obtained from the ATP-$^{32}PP_i$ exchange reaction

(5,6). The dissociation constants for ATP^{4-} and PP_i^{4-} compare excellently with those previously determined using the fluorescence decay method in the presence of urea (1). However, there is disagreement regarding L-isoleucine, presumably reflecting an effect of urea. Mg^{2+} concentrations up to 6.5 mM slightly reduce the quenching for $MgATP^{2-}$ and $MgP_2O_7^{2-}$. When Mg^{2+} was present at high concentrations and in excess of ATP or pyrophosphate, quenching was abolished, presumably because of the formation of di-magnesium salts which do not bind to the enzyme (7).

The shift of the wavelength for maximum emission suggests that binding of a substrate is associated with a conformational change of the enzyme-TNS complex. When excited at 290 nm, energy appears to be transferred from tryptophan side chains to bound TNS. The transfer is not random, as indicated by the higher degree of quenching when excited at 290 nm instead of 366 nm. Presumably, the position of one or more tryptophan side chains is changed relative to the position of bound TNS when a substrate combines with the enzyme.

Conclusion. TNS is suitable as a reporter group since only the fluorescence and not the binding of the ligands nor the ATP-$^{32}PP_i$ exchange activity of the enzyme is perturbed. The technique requires only very small amounts of enzyme; in our hands, concentrations of 5 µg/ml were sufficient.

We are grateful to Mrs. Anne Orme and Mrs. Marie Hebert for their technical assistance and for the purification of the enzyme.

REFERENCES

(1) Penzer, G. R., Bennett, E. L., and Calvin, M., Eur. J. Biochem., 20, 1 (1971).
(2) McClure, W. O. and Edelman, G. M., Biochemistry, 5, 1908 (1966).
(3) Job, P., Ann. Chim., Ser. 10, 9, 113 (1928).
(4) Benesi, H. A. and Hildebrand, J. H., J. Amer. Chem. Soc., 71, 2703 (1949).
(5) Bergmann, F. H., Berg, P. and Dieckmann, M., J. Biol. Chem., 236, 1735 (1961).
(6) Cole, F. X. and Schimmel, P. R., Biochemistry, 9, 480 (1970).
(7) Cole, F. X. and Schimmel, P. R., Biochemistry, 9, 3143 (1970).
(8) Baldwin, A. N. and Berg, P., J. Biol. Chem., 241, 831 (1966).
(9) McClure, W. O. and Edelman, G. M., Biochemistry, 6, 559 (1967).
(10) Calendar, R. and Berg, P., In: Procedures in Nucleic Acid Research, ed. by G. L. Cantoni and D. R. Davies, p. 384. New York: Harper and Row (1960).