Fluorescence Lifetimes of the Tryptophan Residues in Ornithine Transcarbamoylase†

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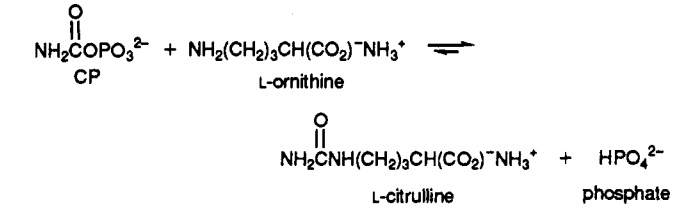
ABSTRACT: Multifrequency (2–230 MHz) phase-modulation fluorescence measurements and site-directed mutagenesis have been employed to assign fluorescence lifetimes, quantum yields, and emission maxima to the four tryptophans in the enzyme ornithine transcarbamoylase from *Escherichia coli* (OTCase) (Trp-125, -192, -233, and -243). OTCase displays two apparent fluorescence lifetimes, 7.2 and 3.2 ns. Results on specific mutants show that Trp-233 has a lifetime of 7.1 ns, while Trp-125, -192, and -243 have lifetimes of 4.0, 3.6, and 4.9 ns, respectively. Thus, the specific conformational changes of the polypeptide segment involving Trp-233 may be monitored conveniently in the wild-type enzyme. On the basis of quantum yield values, Trp-233 is calculated to contribute approximately 43% of the fluorescence intensity of the enzyme, while direct measurements of the enzyme show that up to 65% of the total intensity is really emitted by this tryptophan. The discrepancy may arise from energy transfer from Trp-125 to Trp-233, with an efficiency of 20%. Application of the assigned tryptophan lifetimes to probe ligand-induced protein conformational changes has also been demonstrated.

Fluorescence spectroscopy has been developed into a powerful technique for the investigation of protein conformational changes. However, fluorescence emissions of tryptophans in a protein overlap at all usable wavelengths, and energy transfer occurs among protein tryptophan residues. These often render spectral contributions of individual tryptophans inseparable. Only when one can resolve and assign the fluorescence signals to individual tryptophan residues in proteins can these residues then become valuable, intrinsic probes for monitoring changes in the protein. In this work, multifrequency phase-modulation fluorescence measurements and *in vitro* site-directed mutagenesis are employed to assign fluorescence lifetimes, a quantum yield, and an emission maximum to each individual tryptophan in the enzyme ornithine transcarbamoylase (OTCase) (EC 2.1.3.3).

The *Escherichia coli* K-12 OTCase possesses a trimeric structure of identical subunits and catalyzes, *in vivo*, the first step of the urea cycle (Legrain et al., 1972, 1977). It transfers the carbamoyl group from carbamoyl phosphate to L-ornithine and produces L-citrulline. The binding of substrates and release of products is sequential, with carbamoyl phosphate binding first and L-citrulline released first (Marshall & Cohen, 1972; Legrain & Stalon, 1976; Wargnies et al., 1978).



Several function-related conformational changes of OTCase render it an ideal system for study. Normally, hyperbolic Michaelis–Menten kinetics are observed for both substrates of OTCase from *E. coli* K-12. However, when Zn^{2+} is added to the reaction mixture, the hyperbolic ornithine saturation curve becomes sigmoidal (Kuo et al., 1982; Kuo, 1983). The mechanism of this potential allostery has been studied by using steady-state fluorescence methods (Shen & Kuo, 1987; Lee et al., 1990) and is due to Zn^{2+}-induced conformational changes. Zn^{2+} binds to the enzyme cooperatively. At pH 8.5 and 25 °C, the Hill coefficient of Zn^{2+} binding is found to be 1.6. Enzyme isomerization occurs with a rate constant of 7. Ultraviolet analysis suggests that binding of substrate carbamoyl phosphate (CP) induces conformational changes in OTCase. The CP elicits an isomerization required for the formation of the ternary complex (Miller & Kuo, 1990). The same phenomenon has been reported with yeast OTCase by Eisenstein and Hensley in 1986 (Eisenstein & Hensley, 1986). Another interesting observation is the appearance of allosteric interaction between the enzyme subunits caused by changing a single amino acid residue. The mutation of Arg-106 to Gly-106 induced allostery in *E. coli* K-12 OTCase (Kuo et al., 1989). A similar mutation in a catabolic OTCase, from Glu-106 to Ala-106, caused the enzyme to lose most of its endogenous cooperativity (Baur et al., 1990). Evidence of conformational changes of OTCase has been provided by single-crystal soaking experiments showing that Zn^{2+}, CP, and PALO [*N*-(phosphonoacetyl)-L-ornithine], an analog of the bisubstrate complex, caused the wild-type OTCase

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[1] Abbreviations: OTCase, ornithine transcarbamoylase; Trp, tryptophan; Tyr, tyrosine; Ile, isoleucine; Phe, phenylalanine; CP, carbamoyl phosphate; Orn, ornithine; Nor, norvaline; PALO, *N*-(phosphonoacetyl)-L-ornithine; LB, Luria's broth; dNTPs, mixture of dATP, dTTP, dCTP, and dGTP; Tris , tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; NBS, *N*-bromosuccinimide; ODMR, optical detector magnetic resonance; PAGE, polyacrylamide gel electrophoresis; SI125, SI192, SI233, and SI243 are the singly mutated OTCase. Each of these carries a new isoleucine residue at their replaced sites 125, 192, 233, and 243. SP233 and SP243 each carries a new phenylalanine at sites 233 and 243; D12 (Trp-192 and -233 → Ile) and DI3 (Trp-125 and -243 → Ile) are the doubly mutated OTCase; T125, T192, T233, and T243 are the four triply mutated OTCase. Each of these retains only one tryptophan residue at sites 125, 192, 233, and 243, respectively.

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crystals to crack (Kuo & Seaton, 1989). These results were in good agreement with findings from earlier fluorescence and ultraviolet experiments.

In this work, to further study such function-related conformational changes, fluorescence lifetimes of tryptophan residues were used as intrinsic probes for monitoring changes in the protein. However, OTCase of *E. coli* K-12 contains four tryptophan residues in each subunit (Trp-125, 192, 233, and 243) (Bencini et al., 1983) or a total of twelve tryptophan residues in the native enzyme. Experimentally measured fluorescence signals give a composite or average value over these twelve tryptophan residues.

To associate fluorescence lifetimes with an identified tryptophan residue in a multi-tryptophan protein is difficult. This is not only because lifetimes are usually very short, near a few nanoseconds, but also because one tryptophan often shows more than one lifetime. It is made more complicated also because of possible energy transfer and interactions among the large number of tryptophan residues. This is why most documented fluorescence studies have been limited to proteins containing only one or two tryptophans. Faced with this challenge, the author carried out a study on OTCase mutants made by site-directed mutagenesis. Four single OTCase mutants were made first, by replacing Trp-125, -192, -233, and -243 each separately with isoleucine. Then two double mutants were made by mutating Trp-125 and -243 in one combination and Trp-192 and -233 in another. Finally, using the two doubly mutated genes as templates, four single-tryptophan-containing mutant genes were obtained (these are triple mutants). In addition, in order to examine whether aromatic ring interactions between Trp-233 and Trp-243 are necessary for the functional integrity of the protein, two other single mutants (Trp-233 → Phe, Trp-243 → Phe) were also made. Kinetic characterizations were carried out with each single and triple mutant enzyme to ensure that the enzymes remained functional. The multifrequency (1–250 MHz) phase-modulation technique was used for lifetime measurements. Assignments of lifetimes, a quantum yield, and an emission maximum to each tryptophan in OTCase were successfully made in this research. Finally, the initial application of assigned tryptophan lifetimes to probe ligand-induced protein conformational changes was performed.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The four 25-base oligonucleotides used to introduce site-directed mutation of Trp (TGG) to Ile (ATT) at site 125, 192, 233, or 243, as well as the two 21-base oligonucleotides used to replace Trp (TGG) at site 233 or 243 to Phe (TTT), were prepared with a Milligen synthesizer. The *E. coli* K-12 OTCase gene, *argI*, fragment, which originally was cloned in the plasmid pBR322 in Dr. Wild's laboratory at Texas A&M University (Legrain et al., 1972; Bencini et al., 1983) and later subcloned into the M13mp19 vector in this laboratory (Kuo et al., 1988), was used as the template. The *E. coli* strains *HB1254* (*ara*,Δ*pro-lac*,*thi*/F′*pro*A$^+$B$^+$,*lac*I^q,*lac*ZΔM15,*mut*L::Tn10), which suppressed repairing of the mismatches (Carter et al., 1985), and *TB2* (Δ*arg*I,*pyr*B,*arg*F$^-$), which selected the functional mutants (Legrain et al., 1972), were gifts from Dr. E. Kantrowitz of Boston College. Restriction enzymes and other DNA processing enzymes were purchased from New England Biolabs, Inc. The DNA sequenase kit was purchased from USB Corp. α-[^{35}S]thiodeoxy-ATP was obtained from NEN Research Products. Plasmid pUC18, gene32 protein, and dNTPs were the products of Pharmacia-LKB, Inc. STE-saturated phenol (pH 8.0) and phenol–chloroform were products of Amresco Inc. Ultrapure urea and acrylamide were purchased from Schwarz/Mann Company. The other general chemicals came from Sigma Chemical Company.

Each phosphorylated oligomer (Zoller & Smith, 1982) was annealed to the template. The desired closed-circular reaction products were transformed into competent *HB2154* by the $CaCl_2$ heat-shock method (Davis et al., 1986). The candidate mutants were isolated from picked plaques and verified by sequencing (Sanger, 1981). The mutant genes were then cloned into pUC18 at *Eco*RI and *Hind*III sites. The recombinant products were selected in competent *TB2* cells on minimal M9 plates containing 30 μg/mL of uracil and 50 μg/mL of ampicillin (Wu, 1979). The colonies were grown in LB media with the same concentration of ampicillin for 10–12 h at 37 °C. The cell cultures from each colony were split into two parts. One part was stored at −70 °C as the cell line. The other part was used to confirm the mutation by isolating the plasmid and sequencing the entire gene fragment of 1002 base pairs (Chen, 1985) to ensure that only the expected sites were mutated. A confirmed cell line was chosen for production of the mutant protein. The construction of double and triple mutants was done by using mutant single-stranded DNA as a template and following the same procedure as described above.

Protein Purification. Matrex Blue-A gel was obtained from Amicon; Q Sepharose Fast Flow gel was purchased from Pharmacia LKB product. Cibacron Blue-3GA gel was obtained from Sigma Chemical Company. SDS electrophoresis was used as a criterion for purity of the enzymes (Scopes, 1982). The Bradford method (1976) was employed for determination of protein content. Wild-type OTCase was used to establish a standard curve. The value of the extinction coefficient, 0.96 OD_{280}/mg/mL, for the native OTCase, was reported by Kuo et al. (1988).

Kinetic Properties. Steady-state kinetic methods were used to derive the turnover rates, k_{cat}, and Michaelis constants, K_m, for the wild-type and for each mutant protein. The initial velocities, v_0, were obtained from the rate of production of citrulline, determined by the colorimetric assays of Pastra-Landis et al. (1981) or of Zarabian et al. (1987). The former method was more sensitive and was used as the routine assay for the kinetic data. The latter method was convenient for selecting the desired fractions during protein purification because it gave results within 20 min. In both methods, catalytic reactions were carried out in 50 mM Tris–acetate buffer, pH 8.5, at 25 ± 1 °C. The reaction was quenched after 5 min with 1.0 mL of color mix as described by Pastra-Landis. The concentration of the fixed substrate was 10–15 times the K_m values.

Determination of Tryptophan Contents. The total number of tryptophans and the number of exposed tryptophans in the wild-type OTCase were determined with *N*-bromosuccinimide (NBS) in the presence and absence of urea. Oxidation of tryptophan with NBS was performed according to the method of Spande and Witkop (1967). All titration processes with or without 8 M urea were conducted in 100 mM Tris, pH 7.5, at 20 °C.

Steady-State Fluorescence Experiment. Fluorescence quenching measurements were carried out on sixteen samples of wild-type OTCase in 50 mM Tris buffer, pH 8.5, containing increasing amounts of acrylamide (0–0.8 M). The acrylamide quenching data were analyzed, applying the modified Stern–Volmer equation as described by Lehrer (1971)

$$I_0/(I_0 - I) = 1/([Q]f_aK_{SV}) + 1/f_a \quad (1)$$

where I_0 and I are fluorescence intensities in the absence and

presence of quencher, Q, respectively, K_{SV} is the Stern–Volmer constant, and f_a is the maximum fractional quencher-accessible fluorescence. In the process of dynamic quenching, a plot of $I_0/(I_0 - I)$ vs $1/[Q]$ gives a straight line with the intercept equal to $1/f_a$.

Fluorescence quantum yields of proteins were measured relative to *p*-terphenyl in alcohol. Determinations were made according to the method described by Demas and Crosby (1971). Emission spectra were corrected for the effect of photomultiplier sensitivity and prism dispersion. The quantum yields, Q, were calculated by

$$Q_p = Q_s \frac{A_p OD_s}{A_s OD_p} \quad (2)$$

where s and p refer to the standard and protein solutions, respectively, and where the symbols OD and A designate respectively the optical densities at the excitation wavelength of 295 nm and the area under the fluorescence spectra. The excitation wavelength of 295 nm was used in all fluorescence determinations to ensure selective excitation of the tryptophan residues.

Fluorescence Lifetime Measurements. An SLM 48000S multifrequency phase-modulation spectrofluorometer (SLM Instruments, Inc.) equipped with a graphics plotter and printer as well as a computer was used to measure lifetimes. A solution of *p*-terphenyl in alcohol was placed in the reference cell to correct for 'color error'. A lifetime of 1.05 ns was assigned to the reference solution (Scaiano, 1989). Measurements were made at 20 °C. The temperature of the sample compartment was controlled using an external bath circulator. The sample temperature was measured prior to and after each measurement in the sample cuvette using a digital thermometer (Omega, Model 410 B-TC). The protein samples, about 2×10^{-6} M, were prepared in 50 mM Tris buffer at pH 8.5. Both sample and reference cuvettes had stirring bars to minimize effects of temperature gradients. The excitation light at 295 nm came from a xenon arc lamp, and the emission was observed through a filter with a cutoff wavelength at 320 nm (Scott, WG320) to eliminate Raman scattering. Solutions were less than 0.2 OD at 295 nm. In the initial experiments, using the assigned tryptophan as probes for protein conformational changes, the proteins were first incubated for 2 h with the ligands (Zn^{2+}, 0.1 mM; urea, 8 M; carbamoyl phosphate, 1 mM; or 1 mM for PALO) at 20 °C and pH 8.5. Usually, 14–18 different modulation frequencies were applied in the 2–230-MHz range of the instrument. A nonlinear least-squares data analysis was then used to fit the designed model to determined phase and modulation values. The deviation functions to be minimized were the reduced χ^2, which were calculated with the software programs developed for SLM 48000S.

RESULTS

Site-Directed Mutagenesis. A prerequisite for assignment of fluorescence lifetimes to each tryptophan in the native enzyme spectroscopically is to produce four single-tryptophan-containing OTCase mutants, so that one can unambiguously characterize each tryptophan residue. To accomplish this, three of the four tryptophan residues, in each case, were replaced using site-directed mutagenesis. The design strategy for creating four single-tryptophan-containing OTCase mutants was as illustrated in Figure 1. Following this strategy, a total of twelve mutants was created. Four of these, each carrying a new isoleucine residue, were named SI125, SI192, SI233, and SI243, according to their replaced sites. The other two, each carrying a new phenylalanine, were similarly named

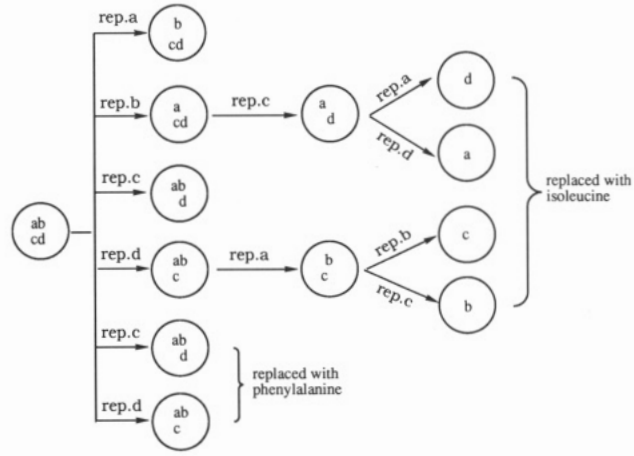

FIGURE 1: Mutation plan. Here, a–d represent the four tryptophan residues located at positions 125, 192, 233, and 243 in OTCase, respectively. Replace with isoleucine, Trp(TGG) → Ile (ATT); replace with phenylalanine, Trp (TGG) → Phe (TTT).

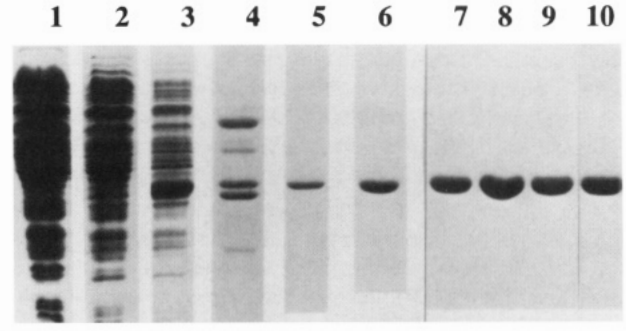

FIGURE 2: SDS polyacrylamide gel electrophoresis of a mutant OTCase, T243, at different stages of the purification process: lane 1, before 40% $(NH_4)_2SO_4$ precipitation; lane 2, after 40% $(NH_4)_2SO_4$ precipitation; lane 3, after 60% $(NH_4)_2SO_4$ precipitation; lane 4, after the Q Sepharose Fast Flow column; lane 5, after the final Blue-3GA column; lane 6, wild-type OTCase as a standard marker; lanes 7–10, PAGE of the four triple-mutant proteins, T125, T192, T233, and T243 (20 μg each).

SP233 and SP243. The two doubly mutated genes were named DI2 (Trp-192 and -233 → Ile) and DI3 (Trp-125 and -243 → Ile), and the four triply mutated genes were designated T125, T192, T233, and T243, corresponding to the remaining tryptophan sites 125, 192, 233, and 243, respectively. All mutants used in this study were confirmed by sequencing the entire OTCase gene.

Purification of Enzymes. Protein purification was a very important step in this work, since some of the mutant proteins have only one tryptophan residue and any minor proteinaceous impurity may have a significant effect on fluorescence lifetime results. Column chromatography on Cibacron Blue-3GA employing varying KCl gradient concentrations and pH values and using multiple columns resulted in purities of isolated mutant OTCases higher than 99% as judged by PAGE. Figure 2 shows SDS polyacrylamide gel electrophoresis (PAGE) of a mutant OTCase, T243, at different stages of the purification process. Lanes 7–10 in Figure 2 display that there are no extra bands observable even with greatly overloaded amounts (about 20 μg each) of four triple mutant OTCases which were the most difficult to purify.

Kinetic Properties. The kinetic properties of the mutated proteins were examined before going on to the fluorescence studies. Table I presents turnover rates, k_{cat}, and apparent K_m values for the wild-type and the four single mutant (Trp → Ile) enzymes. It is seen that replacement of tryptophan with isoleucine at sites 125 and 192 caused almost no changes in catalytic functions. SI125 had a higher K_m^{cp} value than the

Table I: Kinetic Parameters of the Wild-Type and Mutant Enzymes[a]

	k_{cat} (min)$^{-1}$	K_m^{cp} (mM)[b]	K_m^{om} (mM)[b]	K_i^{nor} (mM)[c]
wild type	1.50(0.05) × 10^5	0.040(0.002)	0.40(0.01)	0.054(0.005)
SI125	1.50(0.06) × 10^5	0.12(0.01)	0.45(0.02)	0.046(0.005)
SI192	1.46(0.08) × 105	0.040(0.002)	0.32(0.02)	0.069(0.006)
SI233	4.05(0.15) × 10^3	0.030(0.002)	4.45(0.02)	1.73(0.02)
SI243	2.50(0.1) × 10^4	0.050(0.003)	4.55(0.02)	2.53(0.02)
SP233	8.94(0.16) × 10^3	0.04(0.002)	0.89(0.01)	
SP243	4.60(0.1) × 10^4	0.05(0.004)	1.03(0.01)	
T125	2.70(0.05) × 10^2	0.041(0.005)	3.49(0.18)	
T192	4.40(0.09) × 10^2	0.061(0.005)	4.44(0.12)	
T233	7.65(0.07) × 10^3	0.076(0.007)	5.01(0.2)	
T243	2.32(0.03) × 10^4	0.156(0.007)	8.96(0.24)	

[a] All assays were performed at pH 8.5 and 25 °C. Standard deviation values are given in parentheses. The results reported in this work were based on measurements that were repeated three times. [b] The Michaelis constant K_m values were obtained from initial velocity data of saturation assays. The concentrations of the fixed substrates were 10–15-times K_m values. [c] Inhibition constants of Norvaline were obtained by using L-ornithine as the varied substrate.

wild type had, but their k_{cat} values were identical. This means that the replacement of these two tryptophans with isoleucines had no effect on the catalytic properties of OTCase. However, the replacement of tryptophan by isoleucine at site 233 or 243 reduced the activity of the enzyme significantly and increased the K_m value for ornithine by 10-fold.

It is known that K_m^{orn} values do not measure ornithine binding affinity, since the rapid equilibrium assumption does not hold for wild-type enzyme. One way to estimate the substrate binding affinity is to compare the binding of a competitive inhibitor of this substrate to mutant and to wild-type enzymes (Fromm, 1979). L-Ornithine is competitively inhibited by L-norvaline (Kuo et al., 1988). L-Norvaline structurally resembles L-ornithine, lacking only the δ-amino group. The relatively high values for norvaline K_i^{nor} in Table I thus suggest a weakened ornithine binding of these two mutant enzymes.

The lower ornithine affinities and decreased catalytic rates of SI233 and SI243 might be explained in two ways. First, Trp-233 and Trp-243 might be situated near the catalytic region, especially the ornithine binding domain. Without these residues the mutant could not bind ornithine as well as wild-type OTCase. Alternatively, there might be an interaction between two tryptophan residues through their aromatic rings required for maintaining the conformational structure of the active site that is necessary for catalysis to occur. If so, removal of either one of these two rings would cause some change in the kinetic properties.

To see the possible effect of aromatic ring interactions on kinetic parameters, two other mutants were created with substitution of phenylalanine for tryptophan, one at site 233 and another at site 243. It was found that replacement of tryptophan with phenylalanine changed the K_m values of ornithine (see Table I). The k_{cat} values remained within the same order of magnitude as that of SI233 and SI243. The results are clear that alteration of side chain structures at sites 233 and 243 does not produce major effects on catalytic properties. The obvious similarity between the kinetic data for the two pairs of mutant enzymes, SI233/SI243 and SP233/SP243 supports the validity of these characterizations. So, the changes in kinetic properties of SI233 and SI243 are not due to disrupting of the aromatic ring interaction but most likely are due to their locations near the ornithine binding domain.

Table I also presents the results of kinetic analysis of the four triple mutant enzymes. It is not too surprising to see some decrease in k_{cat} and increase in K_m values greater than those observed in the single mutants, since substitutions for any three of the four tryptophan residues could involve residues Trp-233 and/or Trp-243. In fact, the single-Trp mutants are just the tools in this study; and once the tryptophan probes were selected and characterized, the author focused on the fluorescence changes of the selected probe in wild-type OTCase directly rather than those of the mutants.

Steady-State Fluorescence Measurements. As a result of their high sensitivity to local environments, tryptophan residues in proteins do not fluoresce equally. To assign specific fluorescence contributions to individual tryptophan residues, however, we need information about their immediate environment, fluorescence quantum yields, and fractional intensities. Such information, combined with fluorescence lifetime data, could provide us with more complete fluorescence properties for the four tryptophans in OTCase. Here, several methods, including quenching with acrylamide, *N*-bromosuccinimide titration, and quantum yield measurement, were applied to demonstrate how many tryptophan residues are exposed to the solvent, where they are located, and the fluorescence contributions from the exposed and buried tryptophans.

(*A*) *Fluorescence Quenching.* When the acrylamide concentration was increased from 0 to 0.8 M, fluorescence intensity was reduced and emission maxima gradually shifted toward the blue. The data were analyzed with eq 1. A straight line plot of this equation was fitted to the data of acrylamide titration. It was found that about 80% of the total fluorescence intensity of OTCase was quenched. In other words, about 80% of the total fluorescence had been emitted by exposed tryptophan residues.

(*B*) *Tryptophan Contents.* To determine the total and exposed contents of tryptophan residues in the protein, modification with NBS was used. The number of reactive tryptophan residues per mole of protein was calculated by the equation of Spande and Witkop (1967). The results in the presence of urea therefore show that each mole of OTCase has 11.98 tryptophan residues in total while the results in the absence of urea tell us that six of these are exposed to the solvent. This means that each subunit of OTCase has two exposed tryptophans out of a total of four. The total of four is in agreement with the results from DNA sequencing analysis. As seen later here, the results of fluorescence emission maxima further proved the existence of two exposed tryptophan residues and allowed the identification of these residues.

(*C*) *Fluorescence Quantum Yields and Emission Maxima.* As described in the methods section, the quantum yields, Q, were calculated by using the integrated area under the corrected spectra. These corrected spectra also provided emission maxima, λ_{max}, for these protein samples. Table II presents these quantum yields and emission maxima for the wild-type and triple mutant OTCases. These results show that Trp-125 and Trp-233 have Q values four to five-times higher than those of Trp-192 or Trp-243. Since high quantum yield results in high emission intensity, Trp-125 and Trp-233 appear to be the major emitters in OTCase. According to the relative Q values in Table II, Trp-125 and Trp-233 together should contribute about 80% of the total fluorescence of OTCase.

It is also seen that the emission maxima of Trp-125 and Trp-192 are blue shifted, leading to the conclusion that they are buried inside the proteins, while the maxima of Trp-233 and Trp-243 are red shifted, and thus they are probably exposed to solvents. The conclusion that two tryptophan residues are

Table II: Summary of the Fluorescence Properties of Various OTCase Mutants[e]

	wild type		T125		T192		T233		T243		SP233		W.T. after quenching[d]	
	τ	I%	τ	I%	τ	I%	τ	I%	τ	I%	τ	I%	τ	I%
τ_1[a] (ns)	7.2	65.4	4.0	96.3	3.6	98.2	7.1	96.6	4.9	85.4	4.3	96.5	4.0	88.3
τ_2 (ns)	3.2	34.6	1.0	3.7	0.06	1.8	1.1	3.4	1.0	14.6	0.7	3.5	0.88	11.7
χ^2	0.5		0.5		2.0		1.3		3.7		0.6		0.5	
λ_{max} (nm)	329		322		326		332		331		323		323	
Q[b]	0.4		0.21		0.05		0.26		0.08		0.30			
relative Q[c]			4.2		1.0		5.2		1.6					

[a] p-Terphenyl in alcohol (lifetime τ, 1.05 ns) was the reference solution for proteins. The samples were excited at 295 nm, and the emission was observed through a filter with a cutoff wavelength at 320 nm to avoid Raman scattering. The fractional contribution, I_i%, of each component, τ_i, to total fluorescence was determined at 20 °C, 50 mM Tris, pH 8.5. [b] p-Terphenyl in alcohol was used as the standard solution (quantum yield, 0.93). The excitation wavelength was 295 nm, at 20 °C, 50 mM Tris, pH 8.5. Emission spectra of both protein and standard were corrected. [c] Relative Q values are based on the quantum yield of Trp-192. [d] The wild-type OTCase was first quenched with 0.8 M acrylamide in 50 mM Tris, pH 8.5, before the lifetime determination. [e] All data in this table were carefully checked with samples from different preparations of a protein and also with different determinations from the same preparation of a protein and found to be reproducible.

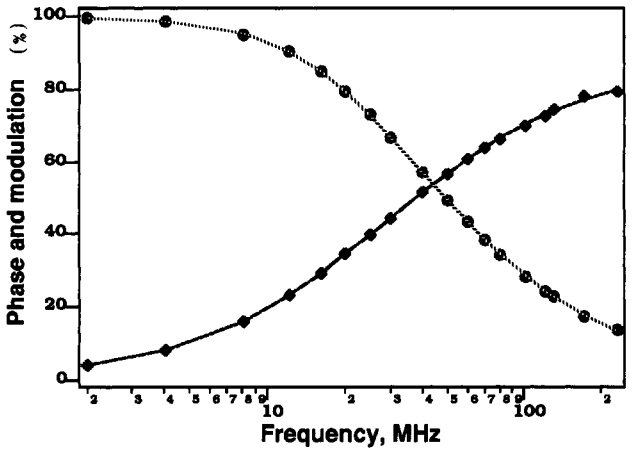

FIGURE 3: Frequency response of OTCase. The solid line is the result of fitting phase lifetime data (◆ symbols). The dotted line is the result of fitting modulation lifetime data (● symbols).

exposed and two buried is in agreement with results obtained from NBS titration experiments.

In summary, OTCase has four tryptophan residues in each subunit. Two are exposed (Trp-233 and -243) and two are buried (Trp-125 and -192). About 80% of the total fluorescence of OTCase is emitted by the two exposed tryptophan residues, and 20% of the total fluorescence of the enzyme is emitted by the two that are buried.

Fluorescence Lifetime Measurements. It should be noted that a spectrofluorometer with multifrequency function is usually able to resolve two lifetime components in a sample if their lifetimes differ by a factor of at least 2 and to resolve three components if the range of their fluorescence lifetimes differs by 10-fold or more (Gratton, 1984). However, the lifetime values of protein tryptophans are not expected to vary by a factor of 10. Nevertheless, the results presented in Table II show that with the aid of site-directed mutagenesis not only could the lifetime values of each tryptophan of OTCase be distinguished but also the long-lived Trp-233 (lifetime, 7.1 ns) appears to be an ideal intrinsic probe for conformational studies.

(A) Wild-Type OTCase. Fluorescence decay of wild-type OTCase is best fitted to a tripple-exponential function. A set of typical fluorescence lifetime data, including frequency-dependent phase and modulation plots, deviation values, and final fitting results is shown in Figure 3. Good resolution is evident from the random deviation between the measured and calculated values and also by the low χ^2 values. Moreover, the data using several different preparations of the enzyme were very reproducible.

The shortest lifetime component, having a contribution of less than 5% of the total intensity, can usually be ignored. Hence, in the following data analysis the two longer lifetime fractional components, I_1 and I_2, are normalized to 100%. About 65% of the total fluorescence is emitted by the component with the lifetime of 7.1 ns. The possibility of having a four-tryptophan protein with fewer than four lifetime values is explained if some of the residues have lifetime values too close together to be resolved by the instrument. Thus, the existence of mutants having a single tryptophan at each of four positions has provided us with a unique opportunity to obtain the lifetime of each tryptophan residue in a multi-tryptophan protein.

(B) Single-Tryptophan-Containing OTCase. The four single-tryptophan-containing mutants of OTCase have been analyzed separately. The results are also listed in Table II. The intensity decay plot of each mutant protein was found to be a double exponential, with a dominant component (85–98%) having a long lifetime and a minor component (2–15%) having a short lifetime. All data in Table II were carefully checked with samples from different preparations of a protein and also with different determinations from the same preparation of a protein and found to be reproducible. For example, T233 was analyzed again after a six-month period with almost identical results. The first measurement produced lifetime values of 7.2 ns (97.3%) and 1.0 ns (2.7%), while the second gave 7.1 ns (96.1%) and 1.2 ns (4.9%).

The two detectable fluorescence lifetimes for each tryptophan residue of the enzyme imply a heterogeneous environment surrounding the tryptophans, which may be interpreted in terms of conformational fluctuations. The large difference between the fluorescence contributions of these two lifetime components tells us that the lifetime value of the long-lived component adequately represents the lifetime value of the tryptophan residue in each of the single-tryptophan-containing OTCases. We therefore conclude that the fluorescences of Trp-125, Trp-192, Trp-233, and Trp-243 are adequately represented by lifetime values of 4.0, 3.6, 7.1, and 4.9 ns, respectively.

From these results, we see that Trp-233 has a much longer fluorescence lifetime than any of the other three tryptophans. Also, it can be resolved definitively from the other lifetimes in wild-type OTCase. In addition, its quantum yield is the highest of the four. Therefore, this tryptophan residue appears to be the best candidate as a probe for conformational changes of the native protein. To confirm that the lifetime value of Trp-233 is 7.1 ns, two more experiments were done. One measured the fluorescence lifetimes of protein SP233, which retains three tryptophan residues and lacks only the residue Trp-233. The second experiment measured the fluorescence lifetimes of wild-type OTCase, which was first quenched with 0.8 M acrylamide. In this way, we not only verified the lifetime

Table III: Effect of Ligands on Fluorescence Parameters of Wild-Type OTCase[a]

	enzyme	+Zn^{2+}	+urea	+CP	+PALO
τ_1 (ns)	7.2	5.2	5.0	6.1	7.2
I%	65.4	83.9	80.0	77.0	67.2
τ_2 (ns)	3.2	1.0	1.2	3.1	3.2
I%	34.6	16.1	20.0	23.0	32.8
λ_{max} (nm)	329	333	348	327	328
Q	0.40	0.15	0.13	0.41	0.41

[a] The concentrations used were as follows: OTCase, 10^{-3} mM; Zn^{2+}, 0.1 mM; urea, 8 M; carbamoyl phosphate (CP), 1 mM; and 1 mM for PALO. The samples were first incubated for 2 h at 20 °C and pH 8.5; then the experiments were conducted at the same temperature.

value of Trp-233 but also determined whether or not Trp-233 was exposed to the quencher. Table II lists the lifetime results as well as the emission maxima in these two experiments. As expected, the absence of Trp-233 resulted in the disappearance of the longer τ, 7.2 ns. The shorter lifetime 4.3-ns component was responsible for more than 96.4% of the emission. Moreover, the emission maximum of SP233 also was blue shifted from 329 to 323 nm. This showed that once Trp-233 was replaced, the fluorescence contribution of the protein was dominated by its second major emitter, Trp-125, which has the lifetime value of 4.0 ns and an emission maximum at 322 nm. The data from this experiment clearly proved that the 7.1-ns lifetime value belongs to the Trp-233 residue and that Trp-233 is on the surface. The same conclusion was supported by the results from the second experiment. After the exposed tryptophans were quenched with 0.8 M acrylamide, the lifetime value of 7.2 ns no longer appeared in wild-type OTCase.

Application of Assigned Lifetimes To Probe the Conformational Changes of OTCase. It was previously reported that OTCase undergoes dramatic conformational changes upon binding Zn^{2+} (Kuo, 1982; Shen & Kuo, 1987; Lee et al., 1990), carbamoyl phosphate (CP), or PALO (Miller & Kuo, 1990). Zn^{2+} binding transforms a noncooperative OTCase into a cooperative one via intersubunit actions. The saturation binding curve of the metal ion is sigmoidal and yields a Hill coefficient of 1.6 at pH 8.5 and 25 °C. In the absence of substrates, Zn^{2+} further promotes a slow enzyme isomerization. The isomerized OTCase is inactive. Thus, the metal is a slow, tight-binding inhibitor. Carbamoyl phosphate also causes a function-related conformational change, but this change enhances the catalytic reaction (Kuo et al., 1988, 1989). PALO binds to OTCase with a K_i of 0.77 μM (Penninckx & Gigot, 1978). A comparison of the difference spectra of the enzyme given by CP alone, by CP and norvaline, and by PALO showed that the PALO-induced difference spectrum is the largest in amplitude (Miller & Kuo, 1990). However, very few details of the contributions of the enzyme polypeptide segments to these different conformations are known. As a result of their high sensitivity to the microenvironment, the tryptophan fluorescence lifetimes of OTCase should allow a more detailed analysis of the conformational flexibility of the enzyme.

From Table III it is seen that besides causing a red shift of the fluorescence maximum from 329 to 333 nm, Zn^{2+} changes the fluorescence lifetimes of the wild-type enzyme from 7.2 and 3.2 ns to 5.2 and 1.0 ns. There are also large changes in fluorescence contributions from the two components as well as a large drop in the quantum yield of OTCase. All these changes, produced by adding Zn^{2+}, are remarkably similar to those caused by adding urea (see Table III). It is well-known that urea can destroy water structure, decrease the hydrophobic effect, and thereby promote the unfolding of protein molecules (Voet & Voet, 1990), whereupon the enzyme becomes inactive. The inactivation of isomerized OTCase induced by Zn^{2+} suggests the possibility that the action of Zn^{2+} on the enzyme might be similar to that of urea. The red shift indicates an increase in solvent exposure of tryptophans upon unfolding. The drop in quantum yield and the changes in lifetimes can be interpreted as due to quenching by solvent in the unfolded state. The only difference between them is that urea caused a bigger red shift of the emission maximum than Zn^{2+} did. Therefore, it is reasonable to say that inactivation of the enzyme upon adding Zn^{+2} is related to partial unfolding.

In contrast, carbamoyl phosphate caused a change in one of the lifetimes from 7.2 to 6.1 ns, and PALO, an analog of the intermediate, caused almost no change in any of the fluorescence lifetimes. The emission maxima and quantum yields were the same as those of the wild type. This means that carbamoyl phosphate, PALO, and Zn^{2+} each affected the conformation of the enzyme in a different way. The reasons for this are not yet clear, but we do know that Trp-233 in the wild-type protein can signal changes caused by adding different ligands. The basic conclusion reached here is that the fluorescence properties of tryptophan are able to reveal specific conformational changes of the enzyme, especially the polypeptide segment containing Trp-233. Such specific information cannot be provided by either UV spectroscopy or enzyme kinetic studies.

DISCUSSION

Site-Directed Mutagenesis and Changes in Enzyme Kinetic Parameters. Site-directed mutagenesis has been popularly used to examine the effects of mutation on enzyme structures and activity. Especially, in the absence of detailed three-dimensional structural information about a protein, this technique is often used to probe which residues are essential for catalysis. When one has obtained a high-resolution crystal structure of a protein, site-directed mutagenesis is still very useful to determine which of the interactions observed in the X-ray structures are functionally important for structural transition (Kantrowitz & Lipscomb, 1990). It is only very recently that this technique has been successfully applied to measure the fluorescence contributions of individual Trp residues in a protein (Waldman et al., 1987). So far there has been no report that point mutation can disrupt the normal conformation of proteins (Creighton, 1990).

In this work, the mutation method was used to produce serial OTCase mutants and assign fluorescence lifetimes for all tryptophan residues in the four-Trp-containing protein. The strategy in replacing Trp residues with isoleucines, and later with phenylalanines, was designed to minimize the perturbation of protein structures and functions. The activity and ligand-binding parameters of the mutant proteins, as listed in Table I, have shown that the protein was adaptive to such site-directed mutations. Even the four triple-mutated proteins were not disruptive and still kept values of k_{cat} and K_m which were quite similar to those of single-mutated enzymes. Also the observed changes are much smaller than those seen in the Arg-57 OTCase mutant (Kuo et al., 1988). Arg-57 is believed to be needed for CP binding and to induce conformational change. The k_{cat} of the Gly-57 mutant was 21 000-fold lower than that of the wild type, and its ornithine binding ability was substantially lower, as estimated by the 500-fold increase in K_i^{nor} (Kuo et al., 1988). Nevertheless, this mutant retained the same overall three-dimensional structure of the wild-type OTCase (Kuo et al., 1988; Kuo & Seaton, 1989). In comparison, the 100- to 1000-fold change in the k_{cat} values and 10- and 20-fold change in the K_m values observed in mutations of this work are relatively small. This means that the tertiary structure of OTCase seems to be the most critical

Table IV: Comparison of ATCase and OTCase in the Binding of Substrates[a]

	ATCase	OTCase
CP	Ser-52, Thr-53, Arg-54, Thr-55, Ser-80, Lys-84, Arg-105, His-134	Ser-55, Thr-56, Arg-57, Thr-58, Ser-81, Lys-86, Arg-106, His-133
aspartate or ornithine	Arg-167, Arg-229, Gln-231, Leu-276	Arg-165, ?, ?, Cys-273, Leu-274

[a] The results are collected from the references Houghton et al., 1984; Volz et al., 1986; Krauseet et al., 1987; and Kuo et al., 1988 & 1990.

aspect for stability of the native conformational state. It is also worthy of note that, to date, no minor changes in kinetic parameters reflecting disruption of the structural integrity of proteins has been reported.

Furthermore, the fact that the replacement of Trp-233 and Trp-243 with isoleucines had the same effect on the catalytic properties of OTCase as the replacement with phenylalanine and the fact that binding abilities for ornithine and for the competitive inhibitor L-norvaline are both weakened all indicate that Trp-233 and Trp-243 might be located near the ornithine binding region. Thus, minor changes of kinetic properties following the mutations involving Trp-233 and Trp-243 are due to decreasing the ornithine binding affinity. Actually, some findings from the study of aspartate transcarbamoylase (ATCase) support this point of view. ATCase shares with OTCase a common evolutionary origin and appears to have 50% functional homology in the primary sequence as well as 75% conservation in the secondary structure (Volz et al., 1986). The residues of ATCase involved in the binding of CP and aspartate have most striking homologies with those of OTCase (Houghton et al., 1984; Krause et al., 1987), as listed in Table IV.

It is clear to see that positions of Trp-233 and Trp-243 in OTCase are very similar to the positions of Arg-229 and Gln-231 in ATCase. These Trp residues appear to be located somewhere within the substrate ornithine binding domain. With this information, it is not surprising to observe the effects of Trp-233 and Trp-243 residues on the kinetic parameters. In addition, Cys-273 has been found recently in our laboratory to affect ornithine binding in OTCase (Kuo et al., 1990), in which the mutation of Cys-273 gave a K_m^{orn} value of 4.2. This is almost the same value obtained from the mutations at Trp-233 and Trp-243 obtained in this work. Therefore, changes in the kinetic parameters of some mutant proteins in this study are considered to be the normal catalytic phenomenon of enzymes. We believe that site-directed mutagenesis is a good tool for fluorescence characterization of multiple-tryptophan proteins. In particular, when those multiple tryptophan residues possess lifetime values and quantum yields quite close to each other, site-directed mutagenesis is the best way so far to identify them.

Energy Transfer among the Trp Residue. The interaction of fluorescence emission within the four tryptophan residues in OTCase is indicated by the following observations:

(1) Trp-233 emits fluorescence more strongly in the wild-type OTCase than in the T233 mutant. According to the relative quantum yields of the four triple-mutant proteins (see Table II), Trp-233, with a τ value of 7.2 ns, is expected to emit about 43% of the total intensity of the wild-type protein if these four tryptophan residues emit independently in the OTCase. However, direct measurement of the wild-type protein showed that about 65% of the total intensity is contributed by this residue, i.e. 20% more than expected.

(2) Proteins with and without Trp-233 show different lifetime values for the remaining three Trp residues. As seen in Table II, wild-type OTCase, which includes Trp-233, has a τ value of 7.2 ns for Trp-233 and 3.2 ns for the others, while SP233, which lacks only Trp-233, has a lifetime value of 4.3 ns. This means that the Trp-233 acts as an energy-transfer acceptor to shorten the lifetimes of some (one or more) of the other tryptophans.

On the basis of these observations of change due to the presence or absence of Trp-233, it is indicated that the tryptophan residues in OTCase do not emit independently. There is probably energy transfer among these residues. If this is so, then the energy gained by Trp-233 must be lost by one of the other three residues. It is of interest to find out which tryptophan residue is the donor that is paired with the acceptor Trp-233. There are three possibilities, two of which can be eliminated as follows:

Trp-243 Is Not the Donor—Our results show that 80% of the total intensity is emitted by the exposed tryptophan residues, Trp-233 and Trp-243. After subtracting the 65% due to Trp-233, the remaining 15% of the quenchable emission can only be attributed to Trp-243. This is consistent with its calculated fluorescence contribution based on quantum yield. This means that in the presence of Trp-233, Trp-243 still emits normally; it does not give up any energy. The donor must therefore be either Trp-125 or Trp-192.

Trp-192 Is Not Strong Enough To Be the Donor—We have already found that Trp-192 is the weakest emitter of the four tryptophans. Its quantum yield value is only 0.05, as shown in Table II. Even if Trp-192 were able to donate its entire energy to Trp-233, it would still not be enough to raise Trp-233 to a high contribution level, i.e. from 44% to 65% of the total intensity of the protein. Therefore, the only possible donor is the remaining Trp-125.

Trp-125 Is the Donor—The common consequences of energy transfer are shortened lifetime and reduced emission intensity of donor. These phenomena were consistently observed with Trp-125. Without the acceptor Trp-233 (e.g. Trp-125 alone), the lifetime value of Trp-125 was 4.0 ns (see data of T125 in Table II). Also, in SP233, which contains three tryptophan residues but not Trp-233, the lifetime value was 4.3 ns and its blue-shifted emission maximum was 323 nm. This result tells us that Trp-125 emits normally and is the dominating emitter even in the presence of Trp-192 and Trp-243. However, in the presence of Trp-233, i.e. in wild-type OTCase, its lifetime of 4.3 ns drops to 3.2 ns, which means that in the excited state Trp-125 loses its energy and returns to the ground state more quickly. Also, in wild-type OTCase, the 3.2-ns-lifetime component contributed about 35% of the total intensity. Of this amount, 15% is due to Trp-243, leaving only 20% to be contributed by Trp-125 and Trp-192. This value is much lower than the 35% expected from Trp-125 according to its relative quantum yield. Therefore, this leads to a hypothesis that energy transfer from Trp-125 to Trp-233 has occurred. Assuming that the only source of additional quenching of Trp-125 in the OTCase is through energy transfer, the transfer efficiency between Trp-125 and Trp-233 can be calculated by the following equation (Lakowicz, 1983).

$$E_t = 1 - (\tau_{da}/\tau_d)$$

Here, τ_{da} is the fluorescence lifetime of Trp-125 in the presence of the acceptor, Trp-233, and τ_d is the lifetime of Trp-125 in the absence of the acceptor. When we use 4.0 ns as τ_d and

3.2 ns as τ_{da}, E_t is approximately 0.2.

Energy transfer between tryptophan residues does not occur in every protein. By using time-resolved fluorescence spectroscopy, Waldman et al. (1987) found that the three tryptophans in *Bacillus stearothermophilus* lactate dehydrogenase did not interact with each other. The lifetime value and fluorescence contribution of each Trp were not changed in either the mutated or wild-type proteins. On the other hand, Eftink (1987) reported from his phase-resolved spectral measurements that strong Trp–Trp energy transfer occurred in a two-tryptophan protein, apoazurin, with a transfer efficiency of 80%. The donor tryptophan had a short lifetime and was buried, while the acceptor tryptophan had a longer lifetime and was on the surface. With a phosphorescence method and ODMR (optical detection of magnetic resonance) spectroscopy, Ghosh (1988) also found Trp–Trp energy transfer in bacteriophage T4 lysozyme. The transfer efficiency depends on the properties of tryptophan residues and experimental conditions. As illustrated by Weber (1970) and further demonstrated by Eftink (1987), Trp–Trp energy transfer with excitation at 280 nm should be higher than that with excitation at the 'red edge', above 295 nm. If OTCase had not had a total of 33 tyrosine residues that might perturb the tryptophan emission when excited at 280 nm, we would have tried exciting the proteins at 280 nm, to see whether or not stronger energy transfer between Trp-125 and Trp-233 occurred. In reality, the four-tryptophan protein could be a more complicated system than that we have discussed above. There is, for example, the possibility of intersubunit energy transfer, which should then be examined; and so, additional future study seems worthwile to provide for greater quantitative detail and a better understanding of any other possible interactions amongst these four tryptophan residues in the wild-type protein.

In conclusion, this work has identified specific fluorescence properties for each of the four tryptophans contained in enzyme OTCase, including quantum yields, emission maxima, site locations, and lifetime values. It has also been demonstrated that Trp-233 is a useful probe for monitoring the conformational changes of OTCase. It is worth mentioning that using native OTCase with this intrinsic probe allays any fear of disturbance of the original protein structure by point mutations. In addition, a hypothesis about energy transfer from Trp-125 to Trp-233 has been put forth.

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