Tryptophan Fluorescence of Chloramphenicol Acetyltransferase: Resolution of Individual Excited-State Lifetimes by Site-Directed Mutagenesis and Multifrequency Phase Fluorometry†

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ABSTRACT: Multifrequency phase fluorometry, in conjunction with site-directed mutagenesis, has allowed the determination of the fluorescence lifetimes of each of the three tryptophan residues of the type III variant of chloramphenicol acetyltransferase (CAT_{III}). The mutant proteins retaining a single tryptophan yield lifetimes of 1.36, 2.00, and 1.17 ns for Trp-16, -86, and -152, respectively. Binding of chloramphenicol shortens the fluorescence lifetimes of all three tryptophans to some extent, in particular those of Trp-86 and Trp-152 (decreases of 51% and 39%, respectively). The mechanism of fluorescence quenching is believed to be radiationless energy transfer. Estimates of Trp−chloramphenicol distances by energy-transfer calculations are in good agreement with those determined from the crystal structure of CAT_{III}. Despite binding at the same site in wild-type CAT_{III}, CoA and ethyl-S-CoA produce different responses in global lifetime measurements—increases of 8% and 31%, respectively. Examination of each of the one-Trp CAT_{III} variants, generated by site-directed mutagenesis, yields a variety of responses. Trp-152, located within the CoA binding site, responds to both CoA and its thioalkyl derivative with a 27−30% increase in fluorescence lifetime. Trp-16, distant from the CoA site, does not differentiate between the two ligands (7% increase in lifetime). However, Trp-86 shows a striking difference in binding responses, only a 4% decrease with CoA but a 14% reduction with ethyl-S-CoA. Each of the two-Trp CAT variants shows little change in global fluorescence lifetime on association with CoA. Although the binding of ethyl-S-CoA produces virtually no change with the W152F variant, significant increases (25−33%) in fluorescence lifetime are observed with each of the two CAT_{III} variants that retain Trp-152 together with either Trp-16 or Trp-86. The measurement of tryptophan lifetimes in two nonproductive ternary complexes allowed the estimation of distances between tryptophan residues and Cm by calculations based on energy transfer. The inferred increase (~6 Å) in distance between Trp-86 and Cm in the ternary complex, compared to that in the binary (CAT•Cm) complex is large and unlikely to be due solely to conformational changes, suggesting that changes in orientation factor and/or the flexibility or rotational freedom of the indole side chain of Trp-86 may be contributory.

Chloramphenicol acetyltransferase (CAT,[1] EC 2.3.1.28) catalyzes acetyl transfer from acetyl-coenzyme A to the 3-hydroxyl of the antibiotic chloramphenicol, an inhibitor of the peptidyltransferase activity of prokaryotic ribosomes [reviewed by Shaw (1983)]. The acetylated product, 3-*O*-acetylchloramphenicol, is unable to bind to ribosomes and is devoid of antibiotic activity (Shaw & Unowsky, 1968).

The type III enzyme (CAT_{III}) is the most catalytically competent of the many naturally occurring variants for which primary structures are known and is the only natural CAT variant for which high-resolution structures are available of both the protein and its binary complexes with ligands. That of the CAT_{III}•chloramphenicol complex is known to 1.75 Å resolution (Leslie, 1990), and the CAT•CoA complex has been determined to 2.4 Å (Leslie et al., 1988). CAT is a trimeric protein (3 × 25 kDa) with identical subunits and three equivalent active sites in clefts at each of the intersubunit interfaces. A major portion of both the chloramphenicol and acetyl CoA binding sites is provided by residues on one subunit, whereas the opposing subunit provides the catalytic histidine residue (His-195).[2] Together, the two substrate binding sites form a 25 Å tunnel through the protein which is fully solvated in the absence of substrates (Leslie et al., 1988; Leslie, 1990). Chloramphenicol and acetyl-CoA approach the catalytic center of each active site from opposite ends of the tunnel, bringing the 3-hydroxyl oxygen of chloramphenicol and the S atom of CoA within 2.8 and 3.3 Å, respectively, of the $N^{\epsilon 2}$ atom of His-195. The independent binding of substrates, first deduced by steady-state kinetic analysis, allows the enzyme to follow a rapid equilibrium ternary complex mechanism with a random order

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of CAT; Cm, chloramphenicol [D-*threo*-1-(4-nitrophenyl)-2-(dichloroacetamido)-1,3-propanediol]; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *p*-cyano-Cm, D-*threo*-1-(4-cyanophenyl)-2-(dichloroacetamido)-1,3-propanediol; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA.

[2] Alignment of the amino acid sequences of many CAT variants (Shaw & Leslie, 1991) has resulted in a general numbering system which is used here. Trp-16, Trp-86, Ser-148, Trp-152, and His-195 are residues 11, 80, 142, 146, and 189, respectively, in the primary structure of type III CAT (Murray et al., 1988).

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of addition of substrates (Kleanthous & Shaw, 1984). The $N^{\epsilon 2}$ of His-195 acts as a general base in the abstraction of a proton from the 3-hydroxyl of chloramphenicol, thus promoting nucleophilic attack of the 3-oxygen at the thioester carbonyl of acetyl-CoA. The resulting tetrahedral intermediate is probably stabilized in the transition state by a hydrogen bond between its oxyanion and the hydroxyl of Ser-148 (Lewendon et al., 1990). In the absence of chloramphenicol, CAT_{III} is able to catalyze the hydrolysis of acetyl-CoA but at a rate that is 10^3-fold slower than that of the transacetylation reaction (Kleanthous & Shaw, 1984).

Previous studies relating the structure of CAT_{III} to the functional properties of the enzyme have addressed many aspects of ligand binding and catalytic efficiency [reviewed by Shaw and Leslie (1991)], including an analysis of the structural basis of intrinsic fluorescence. CAT_{III} contains three tryptophan residues per subunit. Trp-152 is absolutely conserved in all 13 natural CAT variants for which primary structures are available, reflecting the importance of its solvent-accessible position on the surface of the tunnel accommodating the pantetheine arm of CoA. Trp-16 is substituted only once, but Trp-86 is replaced four times, in each case by Tyr or Phe. The side chains of both Trp-16 and Trp-86 are each in a predominantly hydrophobic environment, and both are distant (>16 Å) from the ligand binding sites. The replacement of one, two, or all three tryptophans (by Tyr or Phe) allowed the steady-state characterization of the response of CAT_{III} to ligand binding. Specifically, Trp-86 and Trp-152 were shown to be involved in the fluorescence quenching seen on binding chloramphenicol, with Trp-152 being mainly responsible for the small enhancement (7%) of fluorescence intensity associated with the binding of CoA. A much larger enhancement (25%) was observed upon acetyl-CoA binding, arising mainly from Trp-152 but with contributions from other residues (Ellis et al., 1991a). Clearly, both chloramphenicol and acetyl-CoA are capable of propagating electronic effects over relatively large distances through the protein upon association with it. Fortunately, although catalytic activity is impaired to varying degrees in some of the tryptophan-substituted CAT_{III} variants, substrate affinity is not significantly compromised in any of them (Ellis et al., 1991a).

Valuable though it may be for detecting and quantitating ligand binding, steady-state fluorescence yields only static information, providing time-averaged data on many diverse processes including rotational diffusion, excited-state reactions, and resonance energy transfer. The aim of the current study was to monitor the excited-state lifetimes of the dominant fluorophores of CAT_{III} to characterize individual events occurring within those lifetimes and to identify possible mechanisms by which such long-distance fluorescence changes are generated in both binary complexes and nonproductive ternary complexes.

EXPERIMENTAL PROCEDURES

Purification of CAT_{III}. Purification of wild-type and tryptophan-substituted CAT_{III} variants from *Escherichia coli* extracts was carried out by affinity chromatography on chloramphenicol−Sepharose as described previously (Lewendon et al., 1988). Enzyme preparations were assessed for purity by SDS−polyacrylamide gel electrophoresis with the result that each CAT variant yielded a single band of mobility identical to that of wild-type CAT_{III}. The chloramphenicol used as eluant in the purification procedure was removed by gel permeation chromatography using Sephadex G-50.

The concentration of each of the purified tryptophan-substituted variants of CAT_{III} was calculated by the method of Lowry (1951) using wild-type CAT_{III} as the standard, whereas the concentration of the latter was obtained either by the method of Lowry or by absorbance at 280 nm ($\epsilon_{280}^{1\%} = 13.1$). In each case the reference material was a solution of CAT_{III} of known concentration based on amino acid analysis.

Assay of CAT Activity. CAT activity was assayed spectrophotometrically at 25 °C by a modification of the method of Kleanthous and Shaw (1984). The standard assay mixture contained 1 mM DTNB in TSE buffer, pH 7.5, 0.1 mM chloramphenicol, and 0.4 mM acetyl-CoA. The reaction was initiated by the addition of enzyme, and the rate of formation of CoA was monitored by its reaction with DTNB and liberation of the thionitrobenzoate dianion ($\lambda_{max} = 412$ nm and $\epsilon_M = 13.6 \times 10^3$ M^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount converting 1 μmol of substrate to product per minute under the above conditions.

Preparation of Acetyl-CoA and Ethyl-S-CoA. Acetyl-CoA was prepared by the method described by Simon and Shenin (1953). Ethyl-S-CoA was prepared as described by Lewendon et al. (1994).

Fluorescence Spectra and Quantum Yields. The fluorescence spectrum of each protein was obtained using an SLM 48000S fluorometer with a thermostated cuvette block. Tryptophan fluorescence was selected by excitation at 297 nm using a 200-W Hg−Xe arc source. The emission wavelength was controlled by the monochromator, and an emission bandwidth of 4 nm was used. A typical sample contained 4 μM protein (as CAT monomers) in TSE buffer, pH 7.5, equilibrated to 25 °C. Emission spectra were corrected for photomultiplier sensitivity. Fluorescence quantum yields were measured relative to *p*-terphenyl in alcohol according to the method described by Demas and Crosby (1971) and calculated by

$$\phi_p = \phi_s(F_pA_s/F_sA_p)$$

where s and p refer to the standard and protein solutions, respectively, and F and A designate the area under the fluorescence spectrum and the absorbance at 297 nm, respectively.

Multifrequency Phase Fluorometry. Measurements were performed on an SLM 48000S phase-modulated fluorometer equipped with a 200-W Hg−Xe arc lamp as the excitation source.

Fluorescence Lifetime Measurements. Phase measurements, at 50 frequencies between 1 and 250 MHz, were performed at 25 °C, on each of the wild-type and tryptophan-substituted variants of CAT_{III}. Each ligand was added at a final concentration equivalent to 3−4 times its binary complex dissociation constant (Ellis et al., 1991a; Lewendon et al., 1994), ensuring >75% binding site occupancy while limiting signal loss through the inner filter effect. The excitation wavelength was 297 nm to selectively excite tryptophan residues whereas the emission was observed through a 320-nm cut-on filter. The zero-lifetime reference material was a glycogen suspension sufficiently concentrated to produce a scattering signal comparable to the protein

sample fluorescence. Nonlinear least-squares data analysis was performed to calculate the excited-state lifetime. The reduced $\chi 2$ was the deviation function to be minimized, calculated with the software programs developed for the SLM 48000S.

Energy-Transfer Calculations. The efficiency of depopulation of the excited state by resonance energy transfer (E_T) is calculated from

$$E_T = 1 - (\tau_T/\tau)$$

where τ and τ_T are the excited-state lifetimes in the absence and presence of resonance energy transfer, respectively. Further, the transfer efficiency is directly related to the distance between the donor and acceptor (r) by

$$r = R_0\left(\frac{1 - E_T}{E_T}\right)^{1/6}$$

The term R_0 is that distance at which 50% energy transfer occurs and may be calculated (in centimeters) from (Förster, 1948)

$$R_0^6 = (8.8 \times 10^{-25})(\kappa^2 n^{-4} \phi_D J)$$

where n is the refractive index of the medium and ϕ_D is the quantum yield of the fluorescence donor. The spectral overlap integral (J) of the emission spectrum of the donor and the absorption spectrum of the acceptor was calculated by overlaying the spectral data using the computer program Microsoft Excel.

The term κ is the geometric orientation factor between the acceptor and donor electric transition dipole moments such that

$$\kappa^2 = (\cos \theta_T - 3 \cos \theta_D \cos \theta_A)^2$$

where θ_T is the angle between the emission dipole of the donor and and the absorption dipole of the acceptor and θ_D and θ_A denote the angle between these dipoles and a vector drawn from the midpoint of the CE2/CD2 bond of the donor to the center of the phenyl ring of the acceptor (chloramphenicol), respectively. The transition moment of indole is defined as the line joining NE1 and a point 20% along the CE3/CZ3 bond, as applicable to the ^1L_a indole excited state (Ichiye & Karplus, 1983). The direction of the transition moment of chloramphenicol was assumed to be the line linking C11 to C8 across the phenyl ring (Figure 1). All interatomic distances are those observed in the crystal structure of the CAT·Cm binary complex (Leslie, 1990).

RESULTS

Steady-State Fluorescence of Unliganded Proteins. The protein fluorescence observed upon excitation of CAT_{III} at 297 nm arises almost exclusively from tryptophan residues. This is demonstrated by the fluorescence spectrum of the Trp-less mutant protein, which shows only a small amount of tyrosine fluorescence (Figure 2). In addition, it is important to note that the fluorescence of the wild-type protein may be considered as the sum of the contributions from each of its three tryptophan residues and that this additivity of fluorescence holds true for singly and doubly mutated proteins within the errors expected due to protein

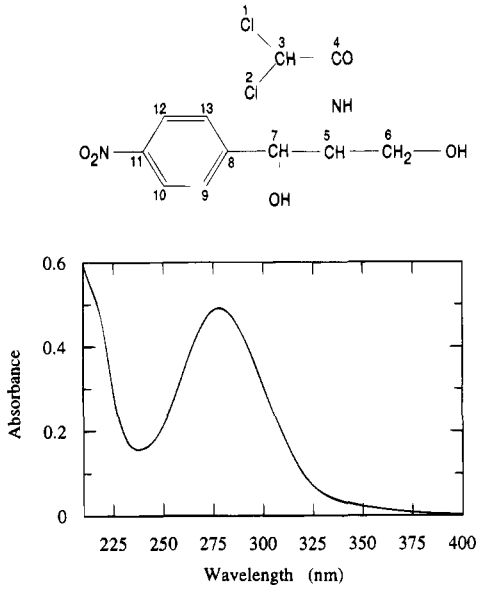

FIGURE 1: Structure and absorption spectrum of chloramphenicol. The numbering system is that used in the CAT_{III}·Cm binary complex data coordinates deposited in the Brookhaven Protein Data Bank.

concentration uncertainty. Such an observation implies that there is no radiationless energy transfer between the tryptophan residues in the wild-type protein and also that there are no detectable conformational changes at any tryptophan residue resulting from the substitutions made at the other tryptophan sites. Furthermore, there is no evidence from the near-ultraviolet circular dichroism spectra of the proteins for conformational changes (R. H. Pain, A. Pickard, J. Ellis, and W. V. Shaw, in preparation).

The corrected spectra of those proteins retaining only Trp-16 or Trp-86 show small blue-shifts (to λ_{max} = 326 nm) from the position of the emission peak of wild type CAT_{III} (λ_{max} = 331 nm), indicating the relative hydrophobicity of their local environments and solvent accessibility (17% and 7%, respectively, from crystallographic data). The emission from Trp-152 is red-shifted (to λ_{max} = 337 nm) relative to the wild-type spectrum as expected, given the substantial solvent-accessible surface of the indole ring (38%), which contributes to the surface of the CoA binding tunnel.

The corrected spectra were integrated from 320 nm upward to calculate the quantum yields and overall fluorescence intensity. Comparison of these integrals indicates that Trp-16 contributes 38% to the wild-type intensity and 34% arises from Trp-86 and 27% from Trp-152. The calculated values are reflected in the quantum yields of 0.066, 0.061, and 0.051 for Trp-16, Trp-86, and Trp-152, respectively.

Fluorescence Lifetimes. (A) Unliganded Proteins. Fluorescence lifetimes for wild-type CAT_{III} and tryptophan-substituted variants were calculated from the frequency dependence of the phase measurements of each protein relative to a zero-lifetime (scattering) solution. In each case, a single lifetime fit described the data adequately with the residuals showing no systematic deviation, whereas more complex, multicomponent analysis produced little or no improvement in the fitting parameters. Figure 3 shows a typical data set obtained for the wild-type protein. Analysis of three such plots yields an average fluorescence lifetime of 1.66 ns. The lifetimes of individual tryptophans may not be resolved without recourse to those CAT_{III} variants retaining only one tryptophan residue. Examination of such

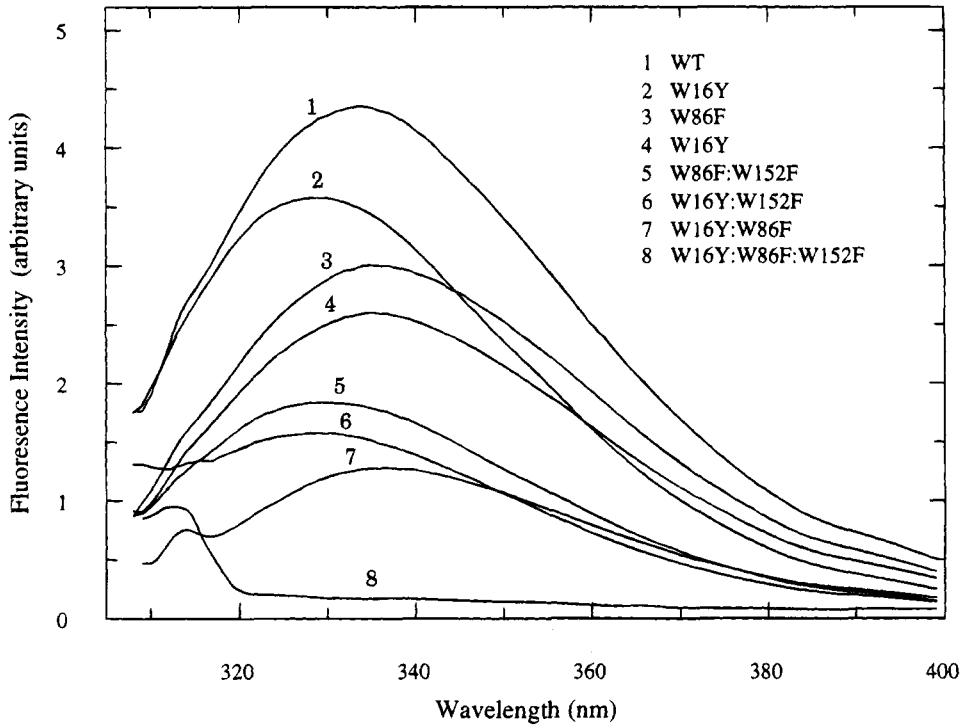

FIGURE 2: Corrected fluorescence emission spectra of CAT_{III} and the tryptophan-deficient variants when excited at 297 nm. Each protein was present at a concentration of 4 μM monomer, in TSE buffer, pH 7.5, equilibrated to 25 °C. The emission bandwidth was 4 nm.

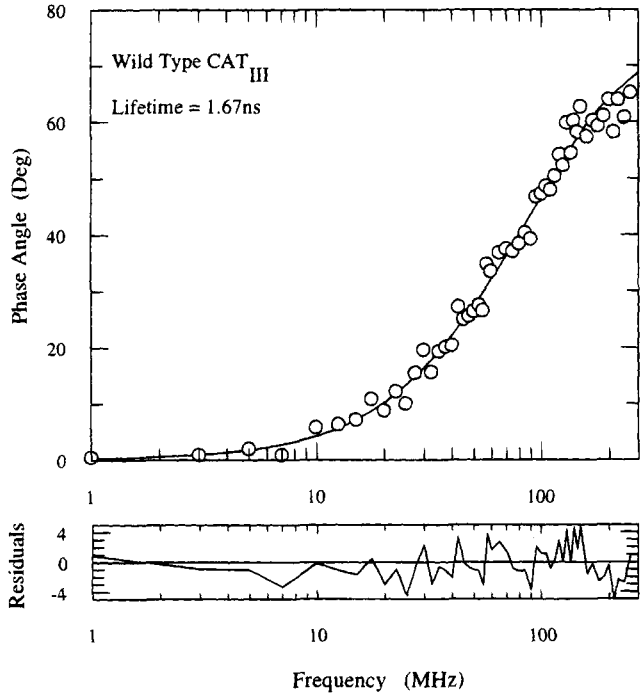

FIGURE 3: Frequency response of wild-type CAT_{III}. A nonlinear least-squares fitting procedure yielded a lifetime of 1.67 ns. The residuals plot shows the difference between the data and the calculated curve.

one-Trp proteins revealed closely spaced excited-state lifetimes ranging from 1.17 to 2.00 ns and inversely related to the solvent-accessible area of the tryptophan side chain responsible (Table 1).

In general, multifrequency spectrofluorometry is unable to resolve three lifetime components within a sample unless those lifetimes differ by an order of magnitude or more (Gratton, 1984). As in the case of CAT_{III}, tryptophan residues within a protein are unlikely to vary to such a degree and it is only with the aid of site-directed mutagenesis that such lifetimes may be resolved. The assumption that the single tryptophan variants give the lifetime of each tryptophan residue as it is in the wild-type protein has been validated in a simulation in which phase data were generated from the lifetimes and contribution of each tryptophan residue as observed in the mutant proteins. This simulated "wild-type" data were then fitted as normal and found to give a single lifetime of 1.53 ns (data not shown), comparing well with the experimental value of 1.66 ns.

(B) CAT·Chloramphenicol Complexes. The binding of chloramphenicol to CAT_{III} leads to a reduction in the excited-state lifetime of each of the tryptophan-substituted variants and the wild-type protein (Table 1). Taken individually, Trp-16 is observed to undergo a 14% decrease in lifetime, whereas Trp-86 and Trp-152 show much greater decreases, 51% and 39%, respectively. All fluorescence lifetimes recover to approximately the values observed for ligand-free protein upon displacement of chloramphenicol from the complex by addition of the substrate analogue *p*-cyano-Cm. This observation is consistent with those made previously (Ellis et al., 1991a) in which the steady-state fluorescence of each of the tryptophan-substituted variants was quenched by the binding of chloramphenicol and dequenched when challenged with *p*-cyano-Cm. In both instances the response originates mainly from Trp-86 and Trp-152, although the greatly enhanced sensitivity of the lifetime measurements suggests a small response from Trp-16, not detected in the steady-state fluorescence.

The contrast in responses due to ligand binding at the chloramphenicol site, a shortening of excited-state lifetimes with chloramphenicol but no change with *p*-cyano-Cm allows the identification of the quenching mechanism. The analogue differs from chloramphenicol only in the replacement of *p*-NO_2 by *p*-CN, with a consequent change in the absorption spectrum from a maximum at 280 nm ($\epsilon_M = 9.6 \times 10^3\ M^{-1}$

Table 1: Fluorescence Lifetimes of Wild-Type and Mutant Chloramphenicol Acetyltransferases

protein	fluorescence lifetime[a] (ns)						
		Cm	Cm + CN-Cm	ethyl-S-CoA	ethyl-S-CoA + Cm	CoA	CoA + Cm
wild type	1.66 ± 0.18	1.05 ± 0.12	1.55 ± 0.14	2.18 ± 0.12	1.12 ± 0.11	1.79 ± 0.15	1.06 ± 0.14
W16Y	1.41 ± 0.12	0.93 ± 0.11	1.45 ± 0.13	1.78 ± 0.15	1.12 ± 0.14	1.53 ± 0.12	0.97 ± 0.13
W86F	1.16 ± 0.09	0.88 ± 0.11	1.20 ± 0.12	1.55 ± 0.11	1.05 ± 0.10	1.21 ± 0.11	0.96 ± 0.11
W152F	1.79 ± 0.11	1.27 ± 0.11	1.80 ± 0.11	1.74 ± 0.15	1.00 ± 0.13	1.75 ± 0.13	0.98 ± 0.12
W86F·W152F	1.36 ± 0.09	1.14 ± 0.14	1.38 ± 0.13	1.45 ± 0.12	1.00 ± 0.12	1.45 ± 0.13	0.99 ± 0.11
W16Y·W152F	2.00 ± 0.16	0.98 ± 0.15	1.92 ± 0.13	1.71 ± 0.14	1.51 ± 0.13	1.93 ± 0.12	1.57 ± 0.12
W16Y·W86F	1.17 ± 0.10	0.72 ± 0.11	0.89 ± 0.13	1.49 ± 0.11	1.00 ± 0.13	1.52 ± 0.13	0.78 ± 0.12

[a] Fluorescence lifetimes shown are the mean of at least three measurements. The error ranges take account of the average phase angle error achieved by the fitting procedure.

cm^{-1}) for Cm to a peak at 234 nm for *p*-cyano-Cm, with no significant absorbance above ~260 nm. A previous study (Ellis et al., 1991a) has shown that CAT_{III} binds and acetylates the two compounds with equal efficiency (as measured by k_{cat}/K_m), and hence, it is unlikely that the difference in fluorescence responses arises from a difference in the mode of binding. Likewise, the propagation of any conformational transitions through the protein arising from ligand binding is unlikely as both compounds would be expected to produce the same response.

The tryptophan lifetime response could arise, therefore, in three distinct ways: (a) static quenching, in which a nonfluorescent ground-state complex forms between fluorophore and quencher, (b) dynamic quenching, requiring collision of chloramphenicol and one or more tryptophan residues, and (c) radiationless energy transfer from tryptophan residues to chloramphenicol. However, static quenching should have no effect on fluorescence lifetime, working instead by reducing the concentration of molecules capable of forming fluorescent excited singlets. Moreover, mechanisms a and b should result in similar responses with both chloramphenicol and *p*-cyano-Cm and also require close proximity of the tryptophan residues and bound substrate. The distance constraint cannot be met because the shortest intermolecular distances between the midpoint of the CD2–CE2 bond of each tryptophan residue and the center of the phenyl ring of the chloramphenicol molecule bound by that subunit are 24.3, 17.2, and 16.6 Å for Trp-16, Trp-86, and Trp-152, respectively (Leslie, 1990). In addition, Trp-16 is only 18.8 Å from the chloramphenicol molecule bound by the adjacent subunit (Figure 4). Taking all of the factors into account, a nonradiative energy-transfer process, as proposed previously (Ellis et al., 1991a), remains the most likely mechanism in operation here.

(C) CAT·CoA and CAT·Ethyl-S-CoA Complexes. The binding of CoA produces small responses in the fluorescence lifetimes of the wild-type (8%) and the two-Trp proteins (−2% to +9%), the slight decrease arising from W152F, implying that Trp-152 is the origin of any increase (Table 1 and Figure 5). Confirmation of this conclusion comes from examination of the single-Trp proteins, which reveal marked differences in responses to CoA binding. The lifetime of Trp-16 CAT is 7% longer and that of Trp-86 CAT 4% shorter, in the complex with CoA compared to that of the free enzyme. Although small, the responses were reproducible. In contrast, the lifetime of Trp-152 CAT is increased by some 30% by the presence of bound CoA.

In two instances, the binding of ethyl-S-CoA (a thioether rather than thioester) by the single-Trp proteins provokes responses similar to those seen with CoA (Table 1 and Figure 5). The much shorter lifetime of Trp-86 CAT with ethyl-S-CoA bound is the exception. Most striking, however, is the fact that all of the CAT_{III} variants containing Trp-152 respond to the binding of ethyl-S-CoA with substantial increases in the duration of the excited state, in contrast to the corresponding CoA complexes, wherein much smaller effects are observed when Trp-152 occurs in combination with any other tryptophan.

The fact that Trp-152 is central to the responses seen on coenzyme binding is most readily explained by its position close to the CoA binding pocket. The side chain of Trp-152, sited at the subunit interface, is approximately 38% solvent accessible in the absence of ligand. When CoA (or its S-substituted derivatives) binds, the displacement of as many as ten ordered water molecules (Leslie et al., 1988) leads to a significant decrease in local polarity, as does the near-coplanar positioning of the adenine ring of the coenzyme with the indole moiety of Trp-152. Once bound, the CP5 atom of the pantetheine arm and the center of the adenine ring are only 5.2 and 8.8 Å, respectively, from the midpoint of the CE2–CD2 bond of Trp-152. The fluorescence response of Trp-152 remains the same whether binding acetyl-CoA (Ellis et al., 1991a), ethyl-S-CoA, or CoA, indicating that there is no significant difference in modes of binding between substrate, inhibitor, and product in the binary complex.

Trp-16 is distant (>14 Å) from the CoA binding site, and the small change in observed excited-state lifetime is independent of the coenzyme analogue used. In contrast, Trp-86 reacts differently to the binding of CoA and its S-substituted derivatives, the latter producing a more pronounced effect on the excited-state lifetime. The distance of Trp-86 from the bound coenzyme (23.3 Å at closest approach) precludes the occurrence of static or dynamic quenching. Furthermore, with no overlap of emission/absorption spectra of tryptophan and CoA, there is no opportunity for radiationless energy transfer to occur. The difference in response may therefore lie in a small structural change (a slight rotation/increased flexibility of the side chain or increase in solvent accessibility) giving rise to an additional route for deexcitation when ethyl-S-CoA is bound compared to the CoA complex.

The behavior of the two-Trp CAT variants and that of wild-type CAT_{III} upon association with CoA can be understood in the light of the above results. When relative intensities are taken into consideration, the overall change observed approximates to the average of the individual tryptophan responses. In particular, the 30% increase in lifetime of Trp-152 is masked by the smaller changes reported by Trp-16 and Trp-86. Ethyl-S-CoA, on the other hand,

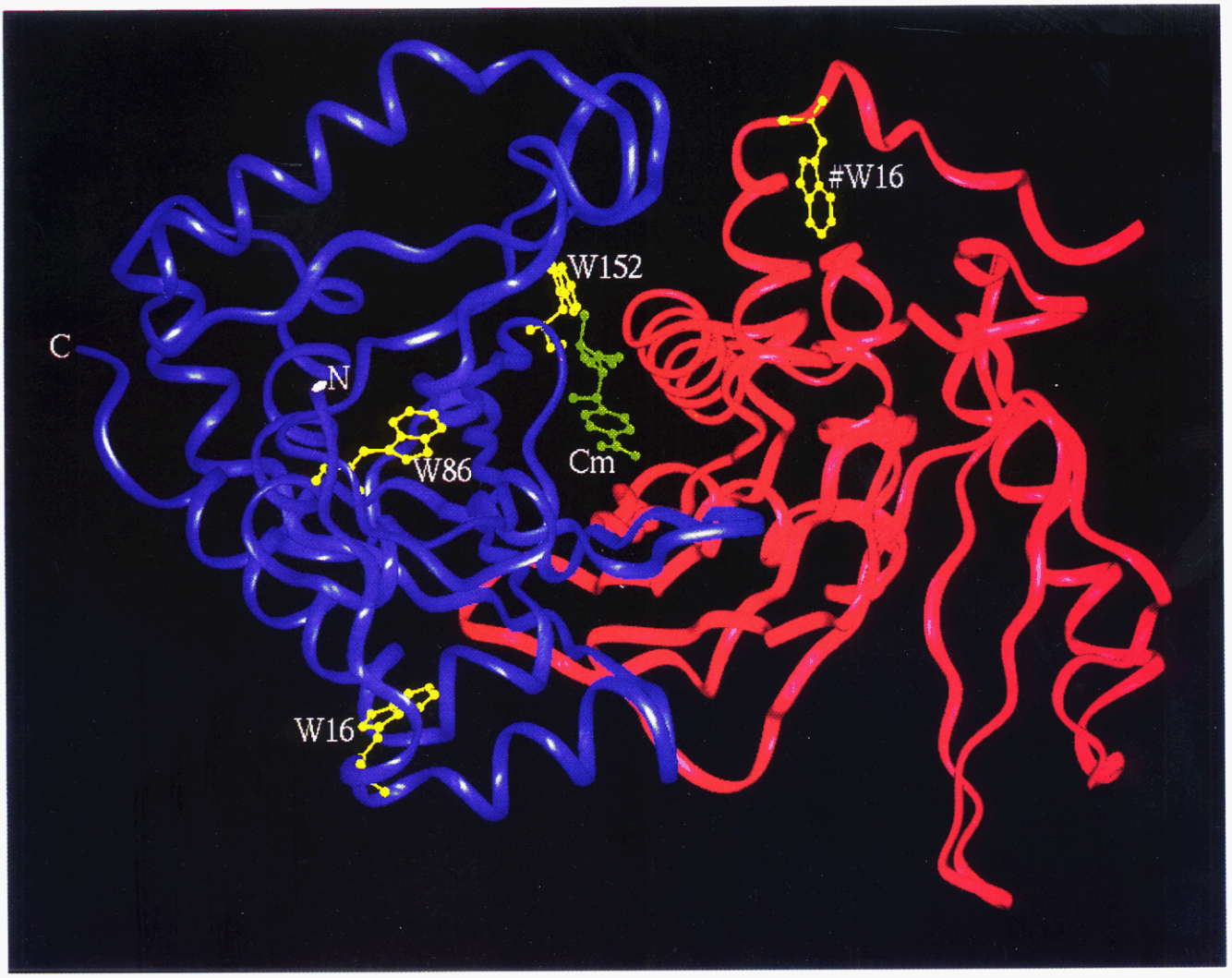

FIGURE 4: Ribbon diagram of two identical subunits of CAT_{III} oriented to show chloramphenicol bound at the interface. All three tryptophan residues of one subunit, together with Trp-16 of the second subunit (denoted by #), are represented by ball-and-stick models.

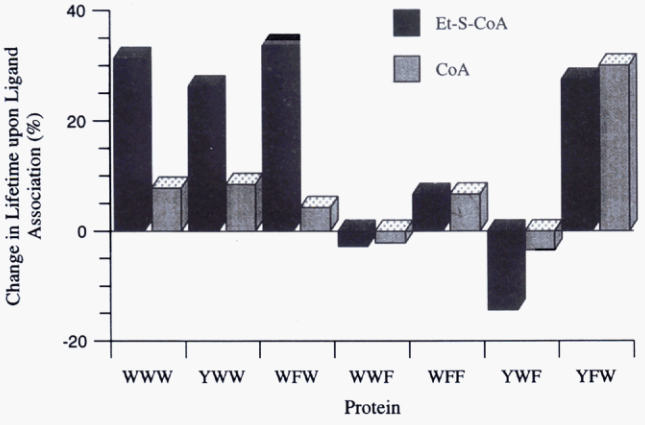

FIGURE 5: Responses of each CAT_{III} variant to the binding of CoA and ethyl-S-CoA. The proteins are labeled by the single-letter code according to the residue present at positions 16, 86, and 152, respectively.

apparently couples the change arising from Trp-152 to those of the other tryptophan residues such that an increase of 28–33% is seen in each case. The theoretical values for energy transfer from Trp-16 and Trp-86 to the closest Trp-152 in the trimer, based on the calculated R_0 and distances observed in the crystal structure, are approximately 0.5% and 8.0%, respectively. As such, neither Trp-16 nor Trp-86 is likely to be the main source of the unexpectedly large response.

DISCUSSION

Energy Transfer between Tryptophan–Chloramphenicol Pairs. The radiationless energy transfer observed between bound chloramphenicol and each tryptophan residue of CAT_{III} was utilized as a spectroscopic "ruler" within the CAT trimer for each of the single-tryptophan variants. The apparent Trp–chloramphenicol distances, calculated from energy-transfer efficiency in the respective binary complexes and the orientation factor (κ^2) as measured in the crystal structure, compare favorably with the distances observed in the crystal structure of the CAT·chloramphenicol binary complex (Table 2). However, the proximity of each bound chloramphenicol molecule to Trp-16 arising from two adjacent subunits leads to energy transfer from both, and therefore, calculations represent an average of the two distances. The small amount of tyrosine fluorescence passed by the 320-nm filter adds a maximal possible error of 0.4% in calculating the Trp–chloramphenicol distances. It is worth noting that, when using a κ^2 of 0.67 (the value generally given to the orientation factor in the absence of crystallographic data), the calculated distances were mostly within 15% of those measured in the crystal structure, only rising to 20% error due to the complication of having two Trp-16 residues within energy-transfer range of each bound chloramphenicol (Table 2).

Table 2: Comparison of Trp−Chloramphenicol Distances As Calculated from Energy-Transfer Efficiencies and Measurement within the Wild-Type CAT_{III} Crystal Structure

	tryptophan−chloramphenicol distance calculated from			
		energy-transfer efficiency		
Trp residue	crystal structure of CAT·Cm (Å)	CAT·Cm (Å)	CAT·Cm·CoA (Å)	CAT·Cm·ethyl-S-CoA (Å)
16	24.3	17.9 (19.4)[a]	15.9	15.9
86	17.2	17.6 (14.8)[a]	22.4	23.5
152	16.6	13.7 (14.7)[a]	14.0	15.5
16[b]	18.8	22.2 (19.4)[a]	19.7	19.7

[a] Distance calculated using an orientation factor (κ^2) of 0.67. [b] Residue belongs to the adjacent subunit. The proximity of each bound chloramphenicol molecule to the Trp-16 residue of two adjacent subunits brings about the possibility of energy transfer to either or both of these acceptors.

When the same calculations are applied to the nonproductive ternary complex of Cm with CAT and either CoA or ethyl-S-CoA, the Trp−chloramphenicol distances differ from those observed in the corresponding binary complex (Table 2). In particular, Trp-86 appears to undergo a 5−6 Å increase in distance from the bound chloramphenicol when either CoA or ethyl-S-CoA is present. Trp-16, on the other hand, appears to be closer to the bound chloramphenicol in the ternary complex by 2 Å, also irrespective of which CoA compound is bound. In contrast, Trp-152 shows only a small increase in distance from chloramphenicol when ethyl-S-CoA or CoA binds to form a ternary complex.

Although the striking change observed for Trp-86 might be taken as evidence for a substantial conformational change, induced by the binding of coenzyme in the ternary complex, it is more likely that the orientation factor (κ) has changed, most probably arising through the rotation of the ring system of the donor or acceptor relative to the other. Such an effect may be obtained either through reorientation of the tryptophan side chain or via a shift of chloramphenicol in the ternary complex compared to its orientation in the binary complex. This conclusion is supported by the crystal structures of the binary complexes of CAT with chloramphenicol and CoA, which are virtually superimposable (Leslie et al., 1988). Hence, no major change in conformation of CAT_{III} is likely. Also, modeling the tetrahedral intermediate, based on the structures of both binary complexes and a knowledge of the catalytic mechanism, requires only very small changes in protein side-chain conformations (<0.2 Å) to accommodate the oxyanion. Notably, the proposed tetrahedral intermediate involves a 0.7 Å movement in the position of the 3-hydroxyl oxygen of chloramphenicol, relative to its position in the binary complex (Leslie, 1990; Lewendon et al., 1990). Such a movement could be accompanied by a change in the direction of the dipole moment of chloramphenicol sufficient to produce a change in the orientation factors and, consequently, changes in the efficiency of energy transfer.

It is particularly important to note that, for the protein retaining only Trp-152, the response to CoA binding seen in the binary complex is largely abolished by the presence of Cm in that the lifetime of Trp-152 in the CAT·Cm and CAT·Cm·CoA complexes is very similar. This is not so for the corresponding complexes with ethyl-S-CoA, the ternary complex lifetime reflecting both an increase due to ethyl-S-CoA binding and a decrease due to the energy transfer to Cm. This observation may reflect a small structural difference between the ternary complex involving substrates (in this case the substrate analogue, ethyl-S-CoA) and that of substrate (Cm) plus a product (CoA). It is known from a previous study that, of the two-Trp CAT_{III} variants, only W152F was catalytically impaired to any degree (Ellis et al., 1991a). The 5-fold decrease in k_{cat} noted for this mutant may be related to these changes observed within the ternary complex. Further support for changes within the ternary complex comes from ligand binding studies in which negative cooperativity was observed between Cm and acetyl- (or ethyl-) S-CoA but not with Cm and CoA (Ellis et al., 1991b). That is, the dissociation constants for Cm and acetyl-CoA were each observed to be 3-fold higher in the ternary complex than in their respective binary complexes. However, no change in binding constants was seen between the binary and ternary complexes when Cm was partnered with CoA in the latter. The tentative conclusion was that the decrease in affinity of CAT_{III} for each substrate in the ternary complex, *en route* to the tetrahedral intermediate and the transition state, signaled the importance of the acetyl group in committing the ternary complex to a productive reaction pathway.

In summary, there are sound reasons for concluding that a nonradiative energy-transfer process, as proposed previously (Ellis et al., 1991a), may be the mechanism by which chloramphenicol quenches the intrinsic fluorescence of CAT. The general agreement between the Trp−chloramphenicol distances calculated from the efficiency of energy transfer and those observed in the crystal structure is consistent with the hypothesis. Although energy transfer may occur over distances of 10−50 Å, it is also dependent on the degree of overlap of the emission/absorption spectra of the donor/acceptor pair and the relative orientation of the dipoles. The apparent change in these distances within the ternary complexes probably arises from reorientation of dipoles rather than gross conformational changes.

Nonetheless, the difference in energy-transfer efficiency for each tryptophan residue in binary as compared is ternary complexes suggests that chloramphenicol undergoes a minor change in position upon binding the coenzyme. The latter could be of relevance to the commitment of a ternary complex to the pathway to products (3-AcCm and CoA) and might mirror the 3-fold decrease in affinity of CAT_{III} for its substrates. In addition, the fluorescence response to ethyl-S-CoA and CoA of the CAT_{III} variant W16Y·W86F (retaining only Trp-152) suggests that these ligands bind in the same manner only in the binary complex. In the presence of chloramphenicol, little or no fluorescence response to the binding of CoA is seen, though ethyl-S-CoA binding still evokes a response from Trp-152. This difference in response of Trp-152 to CoA binding may be interpreted as being due to either an alternative binding mode for CoA or increased rotational freedom/flexibility of Trp-152 in the CAT·Cm·CoA ternary complex relative to that of CAT·Cm·Ethyl-S-CoA. Subnanosecond structural fluctuations and the mobility of tryptophan residues vary considerably from protein to protein and are well-documented (Munro et al., 1979). As it is accepted that crystal structures provide only static images of the protein−ligand complexes, there are sound reasons for believing that the study of fluorescence lifetimes in CAT_{III} and the results obtained may also be detecting changes in local flexibility that are of catalytic importance.

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